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From Södra Barnbördshuset, Wenner-Gren Research Laboratory and the Department of Metabolic Research, the Wenner-Gren Institute, Stockholm, Sweden.

Permeability of Human Placenta to Glucose, Fructose, and Xylose.

By

N. G. HOLMBERG, B. KAPLAN, M. J. KARVONEN, J. LIND and M. MALM.

Received 28 January 1956.

The permeability of the placenta to sugars was not long ago considered a purely physical affair; monosaccharides were assumed to diffuse across the placental barrier readily, whereas this was found virtually impermeable for disaccharides. However, the demonstration of fructose in the foetal blood of sheep and other ungulates in concentrations even exceeding that of glucose made a revision of the concepts of placental permeability necessary. In fact, it was shown that an injection of fructose on either side of the placenta of sheep led to a high concentration gradient across the placenta for a long time, whereas corresponding glucose gradients had a tendency to level off rapidly (KARVONEN 1949, HUGGETT et al. 1951).

The following step was to study, whether the same difference between these two sugars applies also to such species in which the foetal blood contains no or only low concentrations of fructose. The same relationship was shown to hold also in the guinea pig. An infusion of glucose into the maternal circulation caused an almost parallel rise of glucose in the foetal blood, and glucose injected on the foetal side caused only a rapidly passing rise of the foetal glucose level, whereas with fructose the levelling off of a difference in concentration was ten to twenty times slower (KARVONEN and RÄIHÄ 1954).

On the other hand, it has been stated that in man, both fructose and glucose go freely from the mother to the foetus (HUGGETT 1954). However, no adequate quantitative comparison of the rates of the placental transfer of these two sugars has yet been reported for human subjects. Therefore, the writers decided to subject this problem to further study, using the constant speed injection technique.

It has been shown in sheep (KARVONEN and LEPPÄNEN, unpublished) and in guinea pigs (KARVONEN et al., unpublished) that the rate of the transfer of xylose across the placenta comes close to that of glucose. D(+)xylose is an aldopentose, and it is hardly metabolized at all in the animal body. Therefore, experiments were also made by using xylose, employing it as a "tracer" analogue for glucose.

Material and Methods.

The material consists of legal abortions. A vaginal hysterotomy was made to the mother, under aether anaesthesia. The umbilical cord was exposed with caution. An injection of a 30 or 40 per cent sugar solution into the left antecubital vein of the mother was started, using a constant speed injection pump. After an injection of ca. 3 minutes, blood samples were drawn from the right antecubital vein of the mother and from the umbilical vein. Further samples were taken after a period of 5 min. or more, while the injection was still continuing, and again after approximately 5 min. The exact time of taking each sample was noted. In glucose experiments, a sample was taken also before the start of the injection.

In other experiments, sugar solutions were rapidly injected into the umbilical vein, and samples were drawn from it after 3, 8, and 13 min.

The total reducing substance in the blood was determined according to SOMOGYI (1945) and NELSON (1944), and fructose using an indol method (KARVONEN and MALM 1955), after deproteinization with barium hydroxide and zinc sulphate (SOMOGYI). D(+)xylose was determined with SOMOGYI's method, after having first adsorbed glucose from the protein free filtrate to washed baker's yeast. One volume of a 1 : 10 suspension of yeast in distilled water was mixed with 5 vol. of filtrate, kept at room temperature for 4 hours, and centrifuged.

Results.

When *glucose* was injected into the mother, the foetal blood glucose rose remaining closely parallel to the maternal glucose level. On the contrary, an injection of *fructose* produced only a slight rise in the foetal fructose level. Such a difference may have

two explanations. Either fructose penetrates the placenta at a slower rate than glucose, or it is so rapidly utilized or converted *e. g.* to glucose in the foetus that appreciable concentrations are not found in foetal blood, in spite of rapid penetration across the placenta.

In order to test which of these two explanations is valid, glucose or fructose was injected into the foetal circulation, and the rate of the disappearance of the injected sugar from the foetal blood was observed. When glucose was injected into the umbilical vein, the rise of the glucose level passed fast. In some of the experiments the glucose level had returned to the maternal level before the first sample was taken, in appr. 3 minutes; in others it had occurred in 8 min., as seen in the following table:

Table I.

No. of exp.	Foetal age weeks	Dose of glucose mg	Foetal glucose above (+) or below (—) the maternal level	
			1st sample	2nd sample
11	19	150	3′±	8′±
12	17	300	3′±	8′±
15	21	450	3′+	8′—
17	17	600	3′50″+	8′45″—

Evidently glucose disappeared very rapidly from the foetal blood.

The concentration of fructose decreased much more slowly after a similar injection of 1.5 ml of 40 % fructose in a foetus of 15 weeks (No. 16). The difference between glucose and fructose was therefore regarded as essentially due to a difference in the permeability of the placenta to these two sugars.

Experiments with d(+)*xylose* gave results which were intermediate to those obtained with glucose and fructose respectively, independently of the direction of the transfer (Table).

Those of the results which lended themselves to quantitative presentation are shown in Table II. The *apparent net transfer rate* (ANTR) is used as a measure of the permeability of a substance. It is analogous to the diffusion coefficient, expressing change of foetal blood sugar level per minute, as caused by a maternal-foetal gradient of unity. The concentrations are here expressed in mg per 100 ml blood. The dimension of the ANTR is min^{-1}.

Table II.

Transfer of glucose, fructose, and xylose across the human placenta.
$\bar{p}$ = mean foetal level. Other symbols are explained by the headings.

No. of exp.	Foetal age weeks	Interval of samples Δt	Change of foetal sugar mg% $p_1 - p_2$	Rate of change mg% per min. $\frac{\Delta Q}{\Delta t}$	Mean maternal level mg% $\bar{P}$	apparent net transfer rate min^{-1} $\frac{\Delta Q}{\Delta t} \times \frac{1}{\bar{P} - \bar{p}}$
Glucose: mother — foetus:						
3	22	3′	20 → 80	20	155	0.19
4	20	4′45″	110 → 170	12.6	180	0.32
»		5′	170 → 200	6.0	212	0.22
Fructose: mother — foetus:						
1	16	10′30″	2.6 → 8.7	0.58	44	0.015
2	17	7′	1.6 → 6.0	0.62	40	0.018
Fructose: foetus — mother:						
16	15	10′	24 → 18	0.6	2	0.032
Xylose: mother — foetus:						
14	19	8′	0 → 19	2.4	27	0.14
18	17	13′	0 → 35	2.7	83	0.04
19	15	5′	5 → 22	3.4	32	0.19
Xylose: foetus — mother:						
7	19	5′	29 → 15	2.8	0	0.13
9	18	5′	196 → 127	13.8	0	0.10
10	17	5′	131 → 75	11.2	0	0.11
»		5′	75 → 45	6.0	0	0.10

The transfer of glucose from foetus to mother is not included in the table. This depends on the rapid fall of the foetal glucose level after a glucose injection; in none of the experiments were two successive determinations obtained, while the foetal level still was above that in the mother. Thus the ANTR could not be calculated exactly. However, a "minimum" value for the ANTR was calculated on the assumption that the second sample was actually representing the fall. In experiment No. 15 this value came to be 0.74 and in exp. No. 17 1.17; both are far above those of the transfer rate from mother to foetus. It is possible that these high ANTR values depend to an essential extent on a disappearance of glucose along extraplacental channels, *i. e.* utilization.

An examination of the figures in the table suggests that the placental transfer of xylose may be slightly slower than that of glucose. The rates observed with fructose are of a still smaller order of magnitude.

Discussion.

Methods of study.

Exact methods are not easily applicable to the study of placental permeability. The determination of the blood levels of a substance in the umbilical venous and in the maternal venous blood, while the concentration of the substance on either side of the placenta is artificially elevated, is only an approach in this direction. For an ideal determination, the levels of the substance in question in the umbilical artery and vein, the mean level on the uterine side, and the umbilical blood flow ought to be known. For obvious technical reasons, the writers were not able to supply all this information in the present series.

When blood samples are drawn from the umbilical vein, the concentrations observed represent the blood leaving the placenta, but not the mean concentration on the foetal side. If the substance in question is crossing the placenta from mother to foetus, the umbilical vein level is higher than the mean level on the foetal side, and vice versa.

Moreover, by using the blood concentrations instead of plasma concentrations no attention was paid to the unequal distribution of the substance between plasma and cells. A difference between maternal and foetal blood in the haematocrit value and/or in the permeability of erythrocytes to the substance studied will cause an error in defining the gradient between mother and foetus.

In a steady state, the presence or absence of a concentration gradient between maternal venous and umbilical venous blood does not by itself prove anything about the permeability of the placenta, except if one independently knows that the foetal organism is unable to synthetize the substance in question: in such a case the presence of a substance in the foetus evidently proves that it must have come from the mother. If, however, some organic compound is found at a concentration in the umbilical venous blood higher than in the maternal venous blood, this difference may have several explanations. The substance may be synthetized in the foetus, and be passing along a gradient from foetus to mother, or again the placenta may actively "pump" a substance against a gradient from mother to foetus. On the other hand, the finding of a substance in foetal blood at a concentration lower than in the mother does not exclude the participation of "active" processes at the placenta.

When some substance has been given to the mother before delivery or exposure of the foetus, the finding of a higher concentration on the foetal side need not depend on a "pumping" action by the placenta, but may have been caused by diffusion from the mother during a period, when the level on the maternal side was more elevated. A more rapid elimination of the substance by the mother than by the foetus or a slower penetration from the foetal than from the maternal side could explain such a situation.

Observation of the concentrations of a substance in foetal and maternal blood, while its concentration is artificially elevated on either side of the placenta, makes a methodically simple approach possible. In this method, the changes in the umbilical venous concentrations are considered as a measure of the substance entering or leaving the foetal space, depending on the direction of transfer, and the maternal venous concentrations are looked upon as a measure of the concentration prevailing on the maternal side of the placenta. (This assumption is, of course, not strictly valid, as pointed out above.)

If a substance is not metabolized at all, the changes in the maternal and foetal blood levels may be used as fairly reliable indicators of placental transfer. However, if a substance escapes the foetal space rapidly by extraplacental channels, *e. g.* by being utilized, like glucose, the picture is distorted. This distorsion can be reduced, if the transfer can be speeded up without a parallel increase in the rate of extraplacental elimination. The observation that the glucose level in the foetus progressively rises during an infusion into the mother in fact shows that the elimination does not keep pace with the increased transfer. A loss of the substance from the foetus through extraplacental channels will make the transfer from foetus to mother appear more than the real rate.

A further error may be caused by the participation of the substance studied in the metabolism of the placenta. This error will distort the results in a similar way as loss through extraplacental channels.

Obviously, all the observations and the considerations presented above deal with net transfer, and not with absolute transfer rates. It is well known from isotope studies that high rates of molecular exchange may occur across the placenta even in the absence of a net transfer.

Placental transfer of glucose, fructose, and xylose.

When glucose was administered as an infusion to the mother, it had in the present series an ANTR to the foetus approximately 10 to 20 times higher than fructose. The slowness of the rise of fructose in the foetal circulation might also be ascribed to a rapid utilization on the foetal side, but this explanation was excluded by the observation that fructose injected into the foetus disappears from the foetal blood much more slowly than glucose.

Though fructose evidently is able to cross the placental barrier, it does not do this nearly as freely as glucose. This finding contradicts the statement by HUGGETT, referred to above. However, there need not be any real controversy in the results. An analysis of the results of the fructose infusion presented by HUGGETT (the infusion was given at term) shows that during the first 20 minutes of the fructose infusion the maternal fructose level had risen to 100 mg% and that of the foetus to 20 mg%, which values give an ANTR of a similar order as in the present series. Later, after exposing the cord, the foetal fructose started to rise more rapidly, but this may have been caused by manipulations leading to a further deviation from the conditions naturally prevailing *in utero*.

The present results agree remarkably well with the results obtained on guinea pigs (KARVONEN and RÄIHÄ; KARVONEN et al.) and on sheep (KARVONEN; KARVONEN and LEPPÄNEN). The ranges of the ANTR of the three sugars studied for human and guinea pig placentae were as follows:

		Glucose	Fructose	D(+)xylose
Human,	M → F	0.19—0.32	0.015—0.018	0.04—0.19
	F → M	?	0.032	0.10—0.13
Guinea pig,	M → F	0.12—0.27	0.004—0.010	0.018—0.097
	F → M	0.06—0.23	0.004—0.022	0.030—0.111

It may be concluded that at least as far as these sugars are concerned, the permeability of the placenta differs little from species to species, when it is expressed as ANTR and thus relates transfer to the physiologically significant blood level, excluding the effect of variation in the size of the foetus. The similarity between species with quite different placental fructose formation suggests that *the mechanism of transport and fructose formation are to a great extent independent of each other.*

WIDDAS (1952) has demonstrated that in sheep the kinetics of the transfer of glucose across the placenta are typical of a carrier mechanism instead of diffusion. This finding has been extended to the permeability of the guinea pig placenta to d(+)xylose by KARVONEN et al. In the present study, however, the arrangement of the experiments did not allow for a direct study of this problem.

The permeability of erythrocytes to monosaccharides shows similar kinetics (LEFEVRE 1954, WILBRANDT 1954, WIDDAS 1954) as that across the placenta. KARVONEN et al. have also demonstrated that the ANTR of a number of monosaccharides across the guinea pig placenta shows the same general order as that reported for human erythrocytes, several of the aldohexoses passing the placenta rapidly and the ketohexoses slowly. These two analogies — kinetics and chemical specificity — suggest that the transfer of monosaccharides across the placenta and across the cell membrane of erythrocytes occurs with the aid of an identical or closely related mechanism.

Summary.

The permeability of the human placenta to glucose, fructose, and d(+)xylose was studied *in situ* in connection with legal abortions. The mother was given an intravenous injection of the sugar at constant speed. Blood samples were taken during the injection from the umbilical vein as exposed through vaginal hysterotomy and simultaneously from the mother. In other experiments, sugar solutions were injected into the umbilical vein, and blood samples were taken repeatedly after the injection.

The results are expressed as "apparent net transfer rates" (ANTR), *i. e.* as the change of umbilical vein glucose, fructose, or xylose level per minute, as divided by the mean mother-foetus gradient along which the change was occurring. The transfer of glucose and xylose proved fast in comparison to that of fructose. The ANTR values are in the same range as the corresponding figures for guinea pig or sheep placentae.

The value of the method and the implications of the results are discussed.[1]

[1] This work was supported by the Association for the Aid of Crippled Children, New York.

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Oxygen Consumption and Active Sodium Transport in the Isolated and Short-Circuited Frog Skin.[1]

By

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The potential across the isolated frog skin bathed with sodium-Ringer's on both sides depends on the oxygen supply to the skin; this has been shown by several investigators and was most carefully studied by FRANCIS and PUMPHREY (1933). As shown by USSING and his associates (1949—1951), the source of the potential across the skin is the active transport of sodium ions from the outside to the inside. The current which is used to short-circuit the skin, and which is found to be equal to the rate of active sodium transport (ΔNa) will therefore depend on the oxygen supply to the skin.

The purpose of the present study has been to investigate the relationship between the amount of sodium which is transported by the skin and the amount of oxygen used for this transport. This was done by measuring the total oxygen consumption and the short-circuit current on the same skin. As only a fraction of the total oxygen consumption is used for active transport of sodium, it was also determined how much oxygen the skin uses when it does not transport sodium. The latter amount was determined by placing a sodium-free solution, *e. g.* distilled water, on the outside of the skin. Subtraction of this blank from the total amount of oxygen consumed gives the net oxygen consumption

[1] Some results of this investigation were presented by H. H. USSING at a Symposium on membrane transport (Wisconsin University, August 1955).

due to active transport of sodium. A justification for this procedure is found in the fact that the ratio between the net oxygen consumption and the rate of active transport is practically constant independent of the absolute rate.

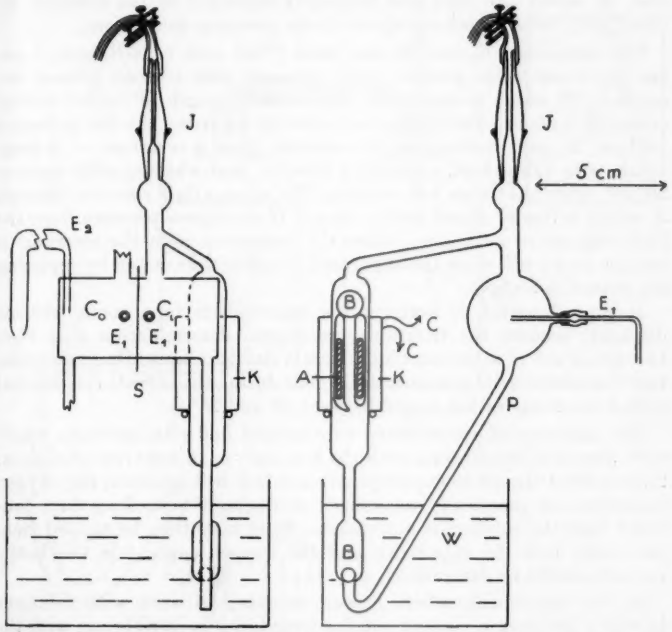

Fig. 1. Apparatus for simultaneous determination of sodium transport and oxygen consumption in the isolated, short-circuited frog skin.

A. iron core
B. glass balls
C_i. inner compartment
C_o. outer compartment
E_1. electrodes for measurement of potential
E_2. electrodes for supplying the short-circuit current
J. ground joint with capillary
K. solenoid
W. water bath
S. skin

Methods.

The apparatus used is shown in figure 1. In principle, it is the same as was used by USSING and ZERAHN (1951), but some modifications have been introduced to avoid the access of air. The apparatus is made almost exclusively of glass, because ANDERSEN and LEHMAN[1] found

[1] Personal communication.

that perspex may give off or absorb oxygen from aqueous solutions. The skin, S, is placed between the two halves of the apparatus, C_i and C_o; these are provided with one pair of electrodes, E_1, for measurement of the potential and another pair, E_2, for supply of the current required for short-circuiting the skin. Stirring is effected by an iron core, A, sealed into glass and frequently attracted by the solenoid, K. Small glass balls, B, act as valves for the pumping mechanism.

The electrodes, E_1 and E_2 are tubes filled with agar-Ringer. J are two interchangeable ground joints (greased with silicone grease) the capillary of which is connected with a short length of rubber tubing closed by a clamp. The filling is effected by removing the two potential bridges, E_1, and conducting the solution from a reservoir — a large bottle with tubulature, capacity 2 litres — and washing with approx. 200 ml, while the stirrer A is working. The surplus fluid runs out through J, which is finally closed with a clamp. If the excess pressure from the fluid column is maintained when the connexion with the reservoir is broken, no air will enter the apparatus, which is then closed by replacing the potential bridges.

It proved useful to perform the experiments in a room without daylight, because the titrations for oxygen determination may then be carried out in a constant light from a daylight lamp. Daily temperature fluctuations did not exceed 0.5°, but during the entire experimental period the temperature varied between 21 and 25° C.

The majority of experiments were carried out with solutions which were almost in equilibrium with the atmosphere. A reservoir of approx. 2 litres filled almost to capacity with solution will maintain the oxygen concentration practically unchanged throughout a working day, provided that the solution is not shaken. Fluid may then be tapped from the bottle into the apparatus, and the oxygen content in the bottle can afterwards be determined.

In the experiments where it was necessary to work with solutions in which the oxygen content was far from being in equilibrium with the atmosphere a different, very simple arrangement had to be used (fig. 2). The bottle with tubulature, 1, contains the solution to be used; the bottle is closed by a one-hole rubber stopper. The air space of bottle 1 is connected with that of bottle 2 by means of glass and rubber tubings. By a similar arrangement the air space of bottle 2 is connected with the water in bottle 3. The solution is saturated with, *e. g.*, nitrogen by passing for 30 to 60 minutes a rapid flow of nitrogen directly from the cylinder through the glass tube, G, the plastic tubing, P, and the stopcock, H, and further through the solution and the bottles. When fluid is afterwards tapped from bottle 1, the oxygen concentration of the fluid changes only very slowly: when this arrangement had remained undisturbed for 40 days, the oxygen concentration had changed from 0.006 ml/l to 1.2 ml/l, *i. e.* 30 μl per litre per 24 hours.

The oxygen concentrations were determined by the Winkler method as modified by KROGH (1935). Samples are withdrawn from bottle 1 by means of a small auxiliary apparatus (fig. 2). When a sample is to be taken, the tip of the Krogh syringe is carried down to the bottom of the

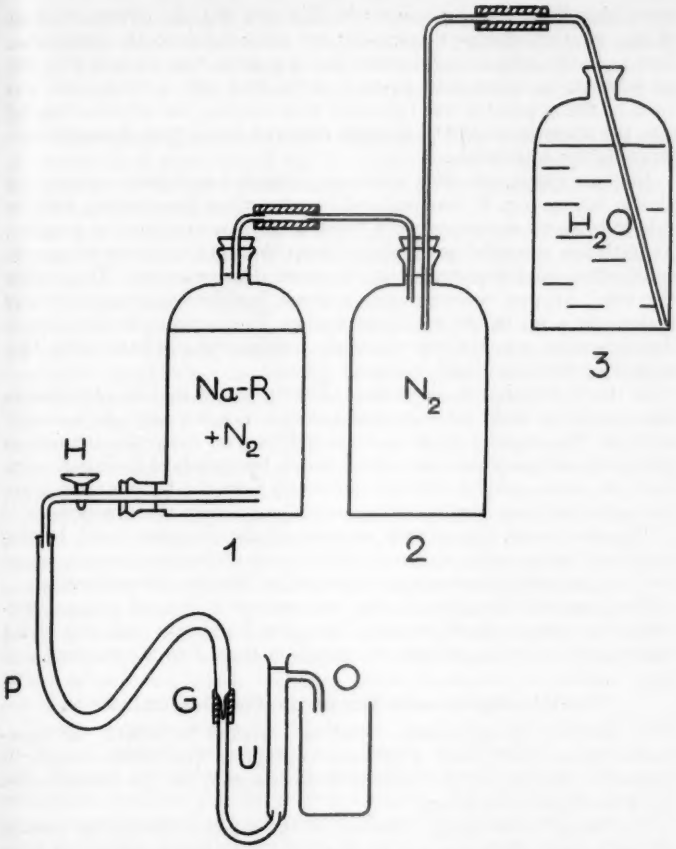

Fig. 2. Apparatus for storage and use of solution in equilibrium with nitrogen.
H. glass stopcock
P. plastic or rubber tubing (in experiments in which oxygen was used instead of nitrogen, P was replaced almost entirely by glass tubing).
U. U tube for withdrawal of samples.

wide branch of the U tube, and the sample is sucked up while a continuous flow of the solution is allowed to escape through the overrun, O.

In order to determine if the oxygen concentration of the solution is changed by being fed from the reservoir to the apparatus, the apparatus was filled with Ringer's in equilibrium with the oxygen of the atmosphere. The oxygen concentrations of samples withdrawn from the apparatus and from the reservoir did not differ significantly. The experiment was repeated with a solution in equilibrium with nitrogen

containing little oxygen; neither in this case did the oxygen content of the solution change measurably by being fed into the apparatus. Just as in the actual experiments, the apparatus was washed with 200 ml solution on either side. Instead of the frog skin a plastic foil was used, or the apparatus was tightened with silicone, the solution was fed into the apparatus and the samples removed for oxygen determination immediately afterwards.

In the experiments with solutions saturated with pure oxygen, the plastic tubing (fig. 2) was replaced by a length of glass tubing, and the rubber-tubing connexions were made as narrow and short as possible. Nevertheless a small loss of oxygen from the solution in the apparatus could often be demonstrated in the control experiments. This makes the total oxygen consumption too large, but does not introduce any systematic error in the net consumption. The method for the oxygen determination was slightly modified, manganous sulphate being first sucked up into the Krogh syringe.

In the description of his method, KROGH stated that he obtained an accuracy of $\pm$ 0.007 ml of oxygen per litre, *i. e.* 1.3 μeq/l. In a sample of 10 ml this would give $\pm$ 0.013 μeq. However, in the present experiments the accuracy was not carried so far, the standard deviation being 0.03 μeq when samples were taken directly from the U tube, and when the solutions were very nearly in equilibrium with the atmosphere.

The error from the oxygen content of the reagents could be disregarded, because the determinations were difference determinations and the reagent volumes were identical in the two determinations.

The standard deviation in the determination of total oxygen consumption was calculated to range from 1 to 3 per cent and that in the determination of net oxygen consumption from 3 to 20 per cent.

Possible Systematic Errors in the Determination.

1. Leaks in the apparatus. This was checked by letting the apparatus remain filled with a solution containing very little oxygen; it appeared that the oxygen content of this solution did not increase even after the lapse of 24 hours.

2. Change in the oxygen content of the solution due to the passage of the current. Experiments to check this possibility showed changes of less than 0.05 μeq per 10 ml solution per experiment at a short-circuit current equivalent to 35 μeq.

3. Possible irregularities in the oxygen consumption, so that the average of the control periods does not give a correct expression of the oxygen consumption when no sodium transport occurs. This source of error may be kept low by discarding the experiments in which the oxygen consumption differs significantly in the two control periods.

4. Bacterial growth in the solution. This is a very serious source of error as it may be expected that the oxygen consumption of the bacteria will increase with their growth and with passage of time. This error may to a great extent be compensated by placing the control periods on either side of the experimental period. However, as it is desirable to avoid growth of micro-organisms, bacterial studies were

carried out.[1] These studies revealed the presence of some unidentified rods, which were sensitive to streptomycin. In all subsequent experiments, streptomycin was therefore added to the solutions in an amount of 0.5 mg per litre. The results of these experiments are marked S in the tables.

5. Production of oxygen in the apparatus. This is unlikely because all experiments were carried out in a dark room with artificial light. Increase in the oxygen concentration was never demonstrated in any of the control experiments.

The measurement of ΔNa was made by reading the short-circuit current. When the solutions are in equilibrium with the atmosphere, this current is equal to ΔNa (Ussing and Zerahn, Koefoed-Johnsen et al., Linderholm, Kirschner, Fuhrmann).

As far as I know, short-circuit experiments have not been carried out with pure oxygen, but it is not very likely that the oxygen concentration should alter the relation between ΔNa and the short-circuit current. In experiments with 95 per cent O_2 and 5 per cent CO_2, both ΔNa and the short-circuit current are reduced to zero because of the fall in the pH level. Accordingly, it was taken for granted that the short-circuit current was equal to ΔNa also in experiments with varying oxygen content of the solutions.

In several of the experiments the outside solution during the control periods (ΔNa = 0) was distilled water. This brings about a change in the osmotic pressure on the outside, but experiments with Mg-Ringer as outside solution showed this to be without any significance.

The use of Mg-Ringer furthermore makes it possible to measure the potential across the skin during the control periods; under these conditions the inside is negative relative to the outside (due to the small efflux of sodium), which indicates active transport of sodium from outside to inside is negligible during the control periods.

Finally, experiments with Mg-Ringer containing 10—20 per cent Na-Ringer were performed in order to study the effect of a concentration gradient for sodium. The sodium efflux resulting from this concentration gradient will to a certain extent disturb the equality of net sodium transport and short-circuit current; but as shown previously (Zerahn, 1955) the efflux amounts to only 0.3—5 μamp./cm^2, and this error is so small compared with the uncertainty of the determination of the oxygen consumption that it may be disregarded.

Results.

Table I shows the results of experiments in which the blank is taken from a period before and a period after the experiments with active transport; during these control periods the bathing solution on the epithelial side of the skin was distilled water. All

[1] The bacterial studies were carried out by Dr. Aksel Stenderup, the Institute of General Pathology, Aarhus.

Table I.

The oxygen consumption of the isolated, short-circuited skin of the frog Rana temporaria with Na-Ringer on both sides of the skin. The net oxygen consumption was found by subtracting the oxygen consumption with distilled water on the outside of the skin.
The solutions were all in equilibrium with the atmosphere. Volume of the apparatus: Before April 1, 1955, 2 × 65 ml; after that date 2 × 31 ml. Area of the skin 7 cm².

S after the date indicates that 0.5 mg of streptomycin per litre of solution was added.

Date	ΔNa μeq hr^{-1}	Oxygen consumption μeq hr^{-1}		in % of ΔNa		ml $gr^{-1}hr^{-1}$ total	Addition to inside fluid
		total	net	total	net		
April 5	5.87	7.16	1.42	122	24.3	0.155	None
April 19	10.00	6.47	2.51	65	25.1	0.163	»
April 22	5.67	5.23	0.81	92	14.3	0.111	»
April 27	11.9	6.62	2.38	56	20.0	0.157	»
August 23 S	8.2	5.40	1.67	66	20.4	0.158	»
August 23 S II..	9.0	5.60	1.77	62	19.7	0.153	»
March 9	10.8	6.95	2.57	64	23.8		Vasopressin
March 28	11.5	7.80	3.20	68	27.8	0.193	»
April 1	6.45	5.16	1.71	80	26.5	0.187	»
April 4	6.51	5.33	1.65	82	25.3	0.118	»
April 26	12.3	7.03	2.65	57	21.6	0.166	»
Mean value	8.94	6.25	1.94	74	22.5	0.156	

the experiments from this series are included, and the dates on which the experiments were carried out are shown.

It is seen that in the majority of the experiments the total oxygen consumption is smaller than the amount of sodium transported.

In many of the experiments vasopressin (mammalian posterior pituitary extract) was added to the inside solution (FUHRMAN and USSING 1950, USSING and ZERAHN 1951). This hormone increases ΔNa and the net oxygen consumption and thus facilitates the determination of the oxygen difference, but it does not seem to exert any influence on the net consumption of oxygen per transported equivalent of sodium.

Table II shows the results of a series of experiments in which the outside of the skin was bathed in Mg-Ringer during the control periods and in Na-Ringer or in Mg-Ringer plus 10—20 per cent Na-Ringer in the experimental period. In these cases, vasopressin was also frequently added to the inside solution.

Table II.

The oxygen consumption of the isolated, short-circuited skin of the frog Rana temporaria with Na-Ringer on the inside and Na-Ringer or Mg-Ringer plus Na-Ringer on the outside. The net oxygen consumption was found by subtracting the oxygen consumption with Mg-Ringer on the outside of the skin. The solutions were all in equilibrium with the atmosphere. Area of the skin 7 cm²; volume of the apparatus 2 × 31 ml.

S after the date indicates that 0.5 mg of streptomycin per litre of solution was added.

Date	ΔNa μeq hr⁻¹	Oxygen consumption μeq hr⁻¹ total	Oxygen consumption μeq hr⁻¹ net	Oxygen consumption in % of ΔNa total	Oxygen consumption in % of ΔNa net	Oxygen consumption ml gr⁻¹hr⁻¹ total	% Na-R outside	Add. ins. fl.
May 27	15.0	7.55	4.05	50	27.0	0.266	100	Vasopressin
» 27	12.1	6.71	2.75	56	22.7	0.117	20	»
June 21	8.2	4.98	1.53	61	18.7	0.121	20	»
» 22	9.7	6.68	2.00	69	20.6	0.122	20	»
» 24	7.4	4.10	1.24	56	16.8	0.137	10	»
» 28	10.1	5.77	1.95	57	19.3	0.128	10	»
August 17S...	4.1	4.85	0.85	118	20.7	0.177	10	»
» 17S II	4.85	5.65	0.73	117	15.1	0.227	10	»
» 19S...	14.9	9.98	2.68	67	18.0	—	100	»
» 19S II	9.0	9.07	1.20	101	13.3	—	100	»
» 24S...	5.60	4.52	0.98	80	17.5	0.127	100	None
» 25S...	9.34	4.75	1.62	51	17.4	0.125	100	»
» 26S...	12.7	7.05	2.82	56	22.2	0.198	100	Vasopressin

When work is performed against a concentration- or potential gradient, it might be expected that the oxygen consumption per transported equivalent of sodium would be larger, but it appears from the table that there was no significant difference.

It should be noted that in many experiments the total oxygen consumption is far below one equivalent per transported equivalent of sodium, and that the net consumption of oxygen is around 20 per cent of the transported amount of sodium, just as in table 1.

Table III gives the results of a series of experiments with varying oxygen tension in the solutions. According to the literature (ADOLPH 1929, FRANCIS 1934), it might have been expected that the transport of sodium would not be essentially influenced by lack of oxygen until the surrounding solutions were in equilibrium with a gas phase containing less than 10 per cent oxygen. However, even in cases of extremely low oxygen tension, it appeared that the net consumption of oxygen did not vary measurably with the oxygen tension.

Table III.

Influence of varying oxygen tensions on the oxygen consumption of the isolated, short-circuited skin of Rana temporaria with Na-Ringer on both sides of the skin, vasopressin added to inside. The net oxygen consumption was found by subtracting the oxygen consumption with distilled water on the outside of the skin. The last column shows the oxygen tension of the gas phase with which the solutions were in equilibrium.

Date	ΔNa μeq hr^{-1}	Oxygen consumption					mm Hg
		μeq hr^{-1}		in % of ΔNa		ml $gr^{-1}hr^{-1}$	
		total	net	total	net	total	
June 10	4.40	2.44	1.38	55	31	0.051	3.3
» 14	7.00	3.61	1.28	51	18.3	0.091	4.6
» 13	7.76	3.05	1.35	39	17.4	0.093	9.1
April 19	10.0	6.47	2.51	65	25.1	0.163	15.5
June 7	12.8	7.01	3.42	54	26.7	0.185	73
October 21S...	11.4	6.90	2.70	61	23.7	0.157	74
» 11S...	10.0	6.90	1.94	69	19.5	0.142	74

Table IV.

The oxygen consumption of the isolated, short-circuited skin of the toad B u f o b u f o. The inside solution was Na-Ringer with vasopressin, the outside solution Na-Ringer.

The net oxygen consumption was found by subtracting the oxygen consumption with distilled water on the outside of the skin. All the solutions were in equilibrium with the atmosphere.

Date	ΔNa μeq hr^{-1}	Oxygen consumption				
		μeq hr^{-1}		in % of ΔNa		ml $gr^{-1}hr^{-1}$
		total	net	total	net	total
April 28	8.6	7.10	2.1	83	24.4	0.194
» 29	11.9	7.48	1.73	63	14.5	0.277
May 2	6.6	6.10	1.48	92	22.4	0.167
September 18....	9.2	7.30	1.28	79	14.0	0.136
Mean value	9.1	7.00	1.65	79	18.9	0.194

Table IV shows the results of some experiments with toad skins. In these skins the net consumption of oxygen was also around 20 per cent of ΔNa. However, the first three experiments shown in this table were carried out with only one control period.

Table V shows the results of experiments with *Rana esculenta*. The net consumption of oxygen is again around 20 per cent of ΔNa.

Table V.

The oxygen consumption of the isolated short-circuited skin of the frog Rana esculenta with Na-Ringer on both sides of the skin. The net oxygen consumption was found by subtracting the oxygen consumption with distilled water on the outside of the skin. All the solutions were in equilibrium with the atmosphere.

Date	ΔNa μeq hr^{-1}	Oxygen consumption					Addition to inside fluid
		μeq hr^{-1}		in % of ΔNa		ml $gr^{-1}hr^{-1}$	
		total	net	total	net	total	
June 16	5.48	3.40	1.86	62	34.0	0.146	Vasopressin
» 17	6.64	4.07	1.18	61	17.8	0.140	»
September 6	8... 17.2	8.90	3.38	52	19.7	0.187	»
» 7	8... 13.6	8.78	3.42	65	25.1	0.260	»
» 8	8... 7.20	6.65	1.07	92	14.9	0.150	None
» 9	8... 7.90	8.30	1.87	105	23.7	0.232	»
Mean value	9.7	6.68	2.13	73	22.5	0.186	

Discussion.

The experiments show that the total amount of oxygen consumed for each equivalent of sodium transported across the skin is often essentially below 1 equivalent. In addition the oxygen consumed when no transport of sodium occurred was invariably smaller than that used when the skin transports sodium. Subtraction of this blank from the oxygen consumption during transport of sodium gives the amount which has here been termed the net consumption of oxygen. It appears that this in all the experiments amounted to about 20 per cent of ΔNa, *i. e.* that only 1 equivalent of oxygen is consumed for each 4 to 5 equivalents of sodium transported.

The question arises, however, whether this net consumption of oxygen is the only source of energy available for the transport of sodium. It is not impossible that some of the oxygen which is used in excess of the net oxygen consumption may give an essential contribution to the transport. However, from table 3 it appears

(1) that the net percentage remains unchanged both when the oxygen tension of the solution is very low, resulting in a marked decrease in the total oxygen consumption, and when the oxygen concentration is increased, and

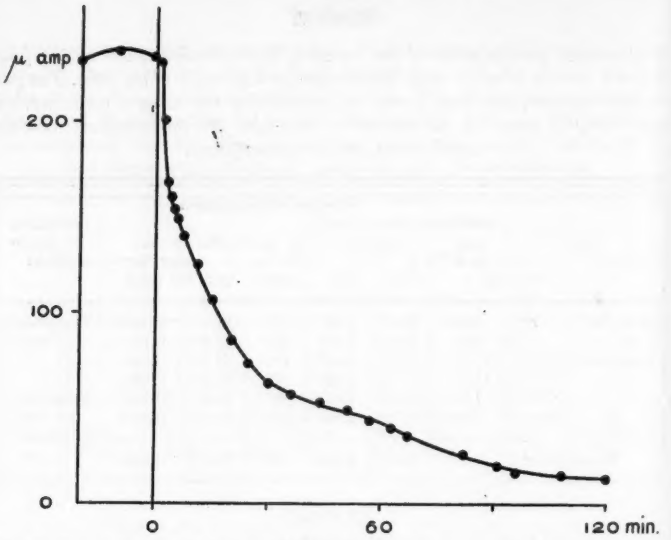

Fig. 3. The influence of oxygen deficiency on the transport of sodium in the isolated short-circuited frog skin.
Ordinate: ΔNa measured by the short-circuit current.
Abscissa: Time in minutes. At zero time the Ringer in equilibrium with the atmosphere was replaced by Ringer in equilibrium with 99.9 per cent nitrogen.

(2) that the total oxygen consumption may be doubled or even trebled without any change in the net percentage.

It might be conceived that energy was derived not only from aerobic, but also to some extent from anaerobic metabolism. The majority of the experiments were performed with solutions which had an oxygen tension in equilibrium with that of the atmosphere. It may therefore be expected that the skin is roughly in a steady state, in which the processes do not shift appreciably relative to each other, and aerobic metabolism must therefore be supposed to be the most essential source of energy. However, it will be of interest to know how much sodium the skin will be able to transport when no oxygen is supplied. This problem was studied by FRANCIS and PUMPHREY (1933), who found that the potential of the skin falls to a low level in the course of 20—40 minutes when it is surrounded by oxygen-free Ringer's solution.

To determine the magnitude of the sodium transport under anaerobic conditions ΔNa was measured when the solutions

bathing the skin were replaced by oxygen-free solutions. The active transport decreased at once and in half an hour fell to a very low value. Figure 3 shows how ΔNa (short-circuit current) changed with the time from the moment when the solutions were changed to oxygen-free Ringer. From this curve was calculated the number of μeq of sodium transported under anaerobic condition in 2 hours. Six determinations gave values ranging from 0.8 to 4.6 μeq, with an average of 2.7 μeq. Similar determinations were made in experiments where aerobic metabolism was stopped by adding KCN to a final concentration of 15 mM. Three determinations gave values for the anaerobic sodium transport ranging from 1.0 to 3.5 μeq, with an average of 2.4 μeq. In the aerobic experiments shown in the tables ΔNa amounted to approx. 10 μeq/hr, and each experiment lasted 2 hours. Anaerobic metabolism will therefore be able to contribute at most 10—20 per cent of the total transport of sodium.

Is the net consumption of oxygen observed large enough to supply the energy required for the transport?

If the calorific value of 1 eq of oxygen (8 g) is fixed at 25,000 gram-calories (dextrose 28,000, lactic acid 23,700 gram-calories) and the net consumption of oxygen at 20 per cent of ΔNa, 5,000 gram-calories will be available for the transport of 1 eq of sodium.

The work performed by the transport mechanism in the transference of 1 eq of sodium may be made up of three components,[1] viz.

1) The work required to overcome the concentration gradient:

$$W_1 = RT\ln(c_i/c_o).$$

2) The work required to overcome the potential gradient

$$W_2 = FE$$

where F is the Faraday constant and E the potential difference.

3) The work required to overcome the internal resistance of the transport system:

$$W_3 = RT\ln(M_{in}/M_{out})$$

where M_{in} is the influx and M_{out} is the efflux (Ussing 1949).

The total work is thus:

a) $$W = W_1 + W_2 + W_3 = RT\ln(c_i/c_o) + FE + RT\ln(M_{in}/M_{out})$$

[1] The equations used to calculate the work performed during transport have been derived by Professor H. H. Ussing, Zoophysiological Laboratory, Department of Biological Isotope Research, University of Copenhagen.

Which can be transformed to

b) $W = F(RT/F\ln(c_i/c_o) + E + (RT/F)\ln(M_{in}/M_{out}))$

converted into common logarithms:

c) $W = F(0.058 \log(c_i/c_o) + E + 0.058 \log(M_{in}/M_{out}))$

which gives the work in joules. If it is wanted to express this in calories, 1 joule = 0.239 calorie is substituted:

d) $W = 0.239 \times 96{,}500\,(0.058 \log(c_i/c_o) + E + + 0.058 \log(M_{in}/M_{out}))$

When the skin is short-circuited, E = 0, and we get:

e) $W = 23{,}100 \times 0.058\,(\log(c_i/c_o) + \log(M_{in}/M_{out}))$ or

f) $W = 1{,}340\,(\log(c_i/c) + \log(M_{in}/M_{out}))$

From equation (c) it appears that

g) $W/F = 0.058 \log(c_i/c_o) + E + 0.058 \log(M_{in}/M_{out}) = E_{Na}$

E_{Na} is the total transport potential for Na defined by USSING and ZERAHN (1951) and may be conceived to be made up of three components in analogy with the work.

If the skin is short-circuited and $c_i = c_o$,

$$W = 1{,}340 \cdot \log(M_{in}/M_{out})$$

The quantity (M_{in}/M_{out}) was not measured, but varies in isolated skins from 10 to 100 (USSING and ZERAHN 1951) and W thus from 1,340 to 2,680 gram-calories per transported equivalent of sodium. The number of calories available were 5,000, so that the percentages used were 26.8 and 53.6, respectively, *i. e.* a sufficient amount of oxygen was present.

If the skin is short-circuited, but $c_i/c_o = 10$, a value for (M_{in}/M_{out}) may be found in the literature; according to the curve in USSING's paper of 1949 it is estimated to be approx. 5.

$$W = 1{,}340\,(\log 10 + \log 5) = 1{,}340\,(1 + 0.7) = 2{,}300 \text{ cal.},$$

which represent 46 per cent of the 5,000 gram-calories available.

If we assume that the energy is provided via adenosine triphosphate (ATP), and that each gram-atom produces 3 energy-rich phosphate bonds of 11,000 calories each, this gives 33,000 calories per gram-atom of oxygen.

If the net consumption of oxygen is fixed at 1/5 eq per 1 eq of transported sodium, 10 eq of sodium will be transported by the energy from 3 gram-atoms of ATP, *i. e.* the Na/ATP ratio is

approx. 3. If all the ATP energy can be utilized for transport of sodium, equation (g) gives:

$$E_{Na} = W/F = 3{,}300/23{,}100 = 0.143 \text{ volt.}$$

This figure may be compared with the 0.110 volt found by USSING and ZERAHN (1951).

Comparative measurements of the oxygen consumption and sodium transport may be performed without difficulty when there is no concentration gradient but only an electric gradient against the transport, but in this case ΔNa must be determined directly by measuring the influx by means of a radioactive sodium isotope.

An accurate determination of the oxygen consumption relative to the sodium transport against high electrochemical gradients is, however, difficult, because ΔNa will be small and the corresponding net oxygen consumptions therefore difficult to determine with a sufficient degree of accuracy. One determination with an electrochemical potential of 108 mv ($c_i/c_o = 10$; $E = 50$ mv) against the transport gave a net consumption of oxygen 20.7 per cent of ΔNa; this suggests that the transport mechanism is unchanged also when the sodium transport occurs against high electrochemical gradients.

Is the oxygen required for the transport derived from the outside or the inside solution? The present experiments did not provide any answer to this question. Usually a net consumption occurred both from the inside and the outside, but the ratio of these quantities varied from experiment to experiment without any change in the net oxygen consumption per transported equivalent sodium. Some experiments were therefore performed in which oxygen-free solutions were used on one side of the skin and solutions in equilibrium with the atmosphere on the other. These experiments showed that the sodium transport across the skin might be slightly reduced, but continued for hours if only sufficient oxygen was present on the other side. It did not seem to be decisive whether the oxygen supply came from the epithelial side or the corium side. In the course of an experimental period, no appreciable amounts of oxygen passed through the skin; hence the fact that the transport of sodium continues cannot be explained by the presence of oxygen on the other side of the skin. The skin is able to utilize oxygen even when the oxygen tension is very low, and it is thus possible that the so-called oxygen-free solutions may have contained small amounts of oxygen

which were used by the skin; concentrations below 15—30 μl of oxygen per litre cannot be measured.

Are the results obtained here in agreement with those reported in the literature?

Attempts to determine oxygen consumption and sodium transport on the same frog skin have not previously been made. However, STAPP (1947) measured the oxygen consumption on a skin which had been partially short-circuited by an external conductor. At that time it was not possible to correlate oxygen consumption with sodium transport, but only with the current generated by the skin. As the skin was only partially short-circuited, the transport of sodium in such experiments must be larger than the current measured (LINDERHOLM, page 129). The remainder of the sodium transport will be counterbalanced by the amount of chloride ion passing across the skin under the influence of the potential. STAPP found that the skin transported more than 0.60 equivalent (calculated from the current) across the skin for each equivalent of oxygen consumed. A single result of 1.64 was reported, but this was believed to be due to some error in the experiment. STAPP (1947 p. 253) introduced the concept of coulomb efficiency (C.E.) which was defined as

$$\frac{\text{Total coulombs generated by skin}}{\text{coulomb equivalents of total } O_2} \times 100 = \% \text{ Coulomb efficiency}$$

He was surprised at the very high values of C.E., but considered them to be possible.

In an unpublished study, ANDERSEN and LEHMAN compared the oxygen consumption by frog skin when sodium was transported and when this transport was partially abolished by carbon dioxide or urethane. They found that the oxygen consumption was highest when sodium was transported, but their experiments do not exclude that inhibition of the oxygen uptake may have occurred by an effect on cells or mechanisms other than those involved in sodium transport. Their results are in agreement with those of PONDER and MACLEOD (1937) and TAYLOR (1935).

The results of previous studies on the oxygen uptake by the frog skin were reviewed by LINDERHOLM (1952), to whose report the reader is referred.

LINDERHOLM determined the oxygen uptake of a series of skins by the Warburg technique and considered these conditions very similar to those prevailing in the short-circuited skin. As this

Table VI.

The oxygen consumption of the isolated frog skin in ml per gramme of wet weight per hour.

Author	Temperature	Mean value	Range	Species
Ponder and MacLeod	25°	0.236		Rana pipiens
Taylor, A. B.	25°	0.330	0.125—0.580	» »
Francis, W. L.	17°	0.180	0.050—0.400	» temporaria
Andersen and Lehmann	20°	0.094	0.066—0.152	» »
Adolph	20°	0.133	0.040—0.300	» pipiens
Linderholm	21.3°	0.136	0.093—0.200	
Lund and Moorman	21.8°	0.139		» »
This paper table 1...	21—25°	0.156	0.110—0.193	» temporaria
table 2...	21—25°	0.159	0.117—0.266	» »
table 5...	21—25°	0.186	0.140—0.260	» esculenta

assumption appears reasonable, it will suffice to mention the results of his short-circuit experiments.

Linderholm found that the oxygen consumption, converted into ml per gramme of wet weight, ranged from 0.093 to 0.200 and averaged 0.136. These figures are in agreement both with the values reported in the literature and those obtained in the present study. He compared the results of these oxygen determinations with the average sodium transport observed in a series of his short-circuit experiments and found a C.E. of 82 per cent. Like Stapp, he considered this result surprisingly high but nevertheless possible, as the sodium pump may be conceived to work with 100 per cent efficiency. However, it cannot be said that Linderholm's results disagree with those of the present study, but the interpretations do. In the first place, Linderholm used average values. Usually both oxygen consumption and sodium transport show wide variations from skin to skin. Several of the skins will not have worked with as high C.E. values as 82 per cent. In order to obtain the high average the C.E. of other skins probably must have exceeded 100 per cent.

Secondly, his material differs from the present in that the latter did not include skins with very low sodium transport. This is due to the fact that posterior pituitary hormone was intentionally used in the present series.

Further studies on oxygen consumption are summarized in table 6. Most of these oxygen determinations were made by the

Warburg technique, and it is therefore not possible to distinguish between oxygen consumption with and without sodium transport. For example, the usual decrease in the oxygen consumption of the entire skin by 3 per cent per hour may be explained equally well by assuming either that the active transport of sodium has decreased or that the metabolism of the entire skin has diminished.

A discussion as to how these results agree with the theories advanced concerning active transport will not be presented here. Suffice it to say that the results disagree with CONWAY's theory, according to which for one molecule of O_2 "The theoretical ratio, if all the electrons received by the oxygen were diverted to Na carriage, is 4.0". (1954 p. 321.)

Finally, it may be of interest to compare the results obtained here with those from muscles and nerves. In these tissues the sodium transport does not cease so suddenly as in the frog skin when the oxygen supply is cut off.

However, it is not unreasonable to assume that when these tissues are in equilibrium with the atmosphere, their energy originates from aerobic processes, and that other sources of energy do not come into action until lack of oxygen occurs.

In frog muscle, KEYNES and MAISEL (1954) found that the ratio of sodium ions transported to oxygen molecules consumed ranged from 2 to 5.7. HODGKIN and KEYNES (1954) reported that transport of 1 equivalent of sodium requires approx. 1 equivalent of oxygen. As this figure is the average of observed values, of which some are considerably higher and other considerably lower than this their investigation points in the same direction as the results of the present study, *i. e.* that it is possible for the organism to perform this transport with an oxygen consumption which is lower than 1 equivalent per transported equivalent of sodium.

Summary.

Studies on the oxygen consumption of the isolated frog skin during active transport of sodium are reported, and the following results were obtained:

1. In most cases, the total oxygen consumption is less than 1 equivalent per equivalent of sodium transported.

2. The amount of oxygen consumed by the skin during transport of sodium exceeds that consumed when no transport occurs.

3. The net consumption of oxygen, equal to the oxygen con-

sumption during transport minus that in the absence of transport, bears a constant ratio to the amount of transported sodium, being 1/4—1/5 equivalent for each equivalent of sodium transported.

4. This ratio is independent of oxygen tension and of treatment with posterior-pituitary hormone, and the same ratio is found with skins from both Rana temporaria, Rana esculenta and Bufo bufo.

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Influence of Varied Levels of Peanut Oil and Cholesterol on Cholesterol and Polyenoic Acids in Tissues of the Chicks.

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The present communication is a continuation of our previous study (DAM, PRANGE and SØNDERGAARD 1955) in which "fat-free" diets and diets containing 10 % peanut oil were compared with respect to their influence on the cholesterol deposition in certain organs resulting from different levels of dietary cholesterol. This study has now been extended to comprise several levels of peanut oil and to include the influence on the distribution of polyenoic fatty acids in the tissue.

Experimental.

One hundred and fourty-four chicks received a "normal diet" (DAM and SØNDERGAARD 1953 (table 2)) from hatching until the 14th day. Thereafter they were distributed into 16 groups (9 chicks in each) and given diets with varying amounts of peanut oil and cholesterol for 4 weeks.

The levels of peanut oil used in the present study were 0, 3, 10 and 20 %. The levels of cholesterol were 0, 0.1, 0.3 and 1.0 %.

The composition of the diets into which the various levels of peanut oil and cholesterol were incorporated (instead of the corresponding weight of sucrose) was the same as the "fat-free" diet no. 3, table 1, in the paper by DAM, PRANGE and SØNDERGAARD (1955). Vitamins

A and D_3 were given in filtrol-treated peanut oil, one drop twice a week per chick, in the amounts indicated in the paper just quoted.

This diet, referred to as "fat-free", contains 0.24 % of fat originating from the crude casein used. Two drops of peanut oil with vitamins A and D_3 contain 47.7 mg of peanut oil.

These minor sources of fat will give an average supply of fat per chick per day of about 24 mg from casein and 7 mg from peanut oil, totalling about 31 mg. This will furnish about $0.4 + 1.4 = 1.8$ mg of dienoic acid per chick per day.

The peanut oil used in the diets was analyzed for polyenoic acids by the isomerization procedure described by HAMMOND and LUNDBERG (1953). It contained the following percentages of polyenoic acids:

Dienoic	21.8 %	Preformed dienoic	0.4 %
Trienoic	0	Preformed trienoic	0.6 %

The filtrol treatment of the peanut oil used as vehicle for vitamins A and D had almost no influence on the percentage of dienoic and trienoic acids.

The fat (0.6 g) extracted from 50 g of the casein by the hydrochloric acid method (SCHMID-BONDZYNSKI-RATZLAFF as described in *Arbejdsmetoder etc.* 1941) was analyzed similarly and found to contain

Dienoic	1.7 %
Trienoic	0.7 %
Tetraenoic	1.0 %

At the end of the feeding period the chicks were sacrificed by decapitation, autopsied, and the following organs taken out for chemical determination:

Liver, subcutaneous adipose tissue (from the left side of the crop), spleen, heart, lungs, brain (cerebrum, cerebellum and medulla oblongata), and aorta (from the right brachio-cephalic to the renal arteries).

The organs, except aorta, were weighed and stored at minus 20° C until they were analyzed (up to 10 months).

The aortae were hardened in 4 % formaldehyde for 2 days, and adhering fat removed by scraping. Then they were dried *in vacuo* at room temperature.

All the organs were treated with alkali and shaken out as described earlier, and the cholesterol content determined by the LIEBERMANN-BURCHARD reaction, except for aorta where the TSCHUGAEFF reaction was used (DAM, PRANGE and SØNDERGAARD 1955). All cholesterol determinations were carried out on individual organs.

Polyene fatty acids (di-, tri-, tetra-, penta- and hexaenoic) were determined in the hypophasic fractions from the separation of the non-saponifiable matter used for cholesterol determinations.

Except in the case of liver, hypophasic phases from the organs of several chicks were pooled, as indicated in table 2.

The alkaline hypophasic fractions were acidified with HCl to pH 1, heated on steam bath for 30 minutes, whereafter the fatty acids were extracted with light petroleum, washed and dried overnight with sodium

sulfate, filtered and taken to dryness *in vacuo* at room temperature.[1] Five mg of the fatty acid sample were used for alkali-isomerization and spectrophotometry as described by HAMMOND and LUNDBERG (1953). Correction for preformed conjugated acids were made ($k = k_{isom.} - k_{preformed}$), and the number of carbon atoms in the pentaenoic acids was arbitrarily considered to be 22. The equations for the percentages of the individual polyenoic acids in the fatty acid mixture were:

$$\text{Dienoic} = 1.09k_{233} - 0.57k_{268} - 0.26k_{315} - 0.12k_{346} - 0.18k_{374}$$
$$\text{Trienoic} = 1.10k_{268} - 0.88k_{315} - 0.02k_{346} - 0.88k_{374}$$
$$\text{Tetraenoic} = 1.65k_{315} - 1.86k_{346} + 0.10k_{374}$$
$$\text{Pentaenoic} = 1.98k_{346} - 1.84k_{374}$$
$$\text{Hexaenoic} = 3.41k_{374}$$

Results and Discussion.

Cholesterol content of tissues, table 1.

The addition of peanut oil without cholesterol did not result in increase of cholesterol in any of the organs examined.

Of these the *liver* is that which has increased its cholesterol content to the highest degree after combined cholesterol and peanut oil feeding. The cholesterol content of the liver increased somewhat even when no fat was added to the diet. This is in accordance with our previous experiments (DAM, PRANGE and SØNDERGAARD 1955) using the same type of casein containing 1.2 % of fat, whereas the use of "vitamin test casein" containing only 0.11 % of fat resulted in a lesser, probably insignificant increase of liver cholesterol. The values for liver cholesterol after feeding of diets containing 0 or 0.1 % of cholesterol were higher when no fat was added than when the diets contained 3, 10 or 20 % peanut oil. With 0.3 % of dietary cholesterol the average values found for liver cholesterol went up as the level of dietary peanut oil increased. However, the values obtained with 10 % and 20 % of peanut oil were not significantly different from each other. With 1 % dietary cholesterol the influence of increasing amounts of peanut oil was very marked.

The *fat tissue* was much less influenced than the liver, but at

[1] In the case of brain it is worthwhile to notice that saponification with alkali is not sufficient to split fatty acids from cerebrosides, whereas treatment with 10 % sulfuric acids leads to complete hydrolysis (THIERFELDER and KLENK 1930). However, cerebrosides are not known to contain polyenoic acids. Further, in our experiments the amount of fatty acids which could be obtained by hydrolysis with 10 % (w/w) sulfuric acid on steam bath for 4 hours, after the above mentioned treatment had been carried out, was only 2 to 5 % of the total amount of fatty acids. Therefore, we consider the procedure adopted to be sufficient for our purpose.

Table

Content of cholesterol in tissues and weight of chicks fed diets with varying 100 g of tissue. The figures for aorta refer to dry

Group no.	1440	1441	1442
Addition to diet: % peanut oil	0	0	0
Addition to diet: % cholesterol	0	0.1	0.3
Liver	384[9] ± 18	433[9] ± 26	519[9] ± 43
Fat tissue	137[9] ± 16	124[9] ± 8	153[9] ± 8
Spleen	429[9] ± 26	431[9] ± 11	473[9] ± 34
Heart	197[9] ± 5	202[9] ± 5	207[9] ± 4
Aorta (dry weight)	770[9] ± 70	770[9] ± 50	910[9] ± 80
Lung	432[9] ± 17	451[9] ± 19	477[9] ± 24
Brain	1,241[9] ± 24	1,212[9] ± 17	1,248[9] ± 20
Average weight of animals in g: initial	89[9]	89[9]	89[9]
Average weight of animals in g: final	218[9] ± 11	241[9] ± 24	264[9] ± 23

Group no.	1448	1449	1450
Addition to diet: % peanut oil	10	10	10
Addition to diet: % cholesterol	0	0.1	0.3
Liver	284[9] ± 11	323[9] ± 10	640[9] ± 129
Fat tissue	129[9] ± 11	117[9] ± 8	156[9] ± 14
Spleen	400[9] ± 24	448[8] ± 13	496[9] ± 19
Heart	190[9] ± 3	188[9] ± 2	233[9] ± 9
Aorta (dry weight)	720[8] ± 40	670[9] ± 30	1,100[8] ± 110
Lung	424[9] ± 24	394[7] ± 27	504[9] ± 28
Brain	1,208[9] ± 25	1,193[9] ± 24	1,222[9] ± 17
Average weight of animals in g: initial	89[9]	89[9]	89[9]
Average weight of animals in g: final	302[9] ± 19	301[9] ± 12	294[9] ± 18

[1] All figures are given as averages of the number of determinations indicated as

least diets with 1 % of cholesterol and 10 or 20 % of peanut oil caused marked increase of cholesterol in the fat tissue.

Spleen, heart and *aorta* were not significantly influenced by 0.1 % dietary cholesterol with any amount of peanut oil tested. 0.3 and 1 % of dietary cholesterol caused increases in cholesterol in these organs, the increases being somewhat larger as the level of dietary peanut oil increased until 10 %. Increase of peanut oil from 10 to 20 % did not result in further increase of cholesterol in these organs.

The cholesterol content of *lungs* was somewhat less influenced than that of the aforementioned organs.

In the *brain* no change of the cholesterol content occurred with any of the dietary regimens examined, an observation which is

1.

amounts of cholesterol and peanut oil for 4 weeks, expressed as mg per weight, those for the other organs refer to wet weight.[1]

1443	1444	1445	1446	1447
0 1.0	3 0	3 0.1	3 0.3	3 1.0
$519^{7} \pm 33$	$297^{9} \pm 14$	$337^{8} \pm 11$	$546^{9} \pm 44$	$1{,}080^{9} \pm 116$
$139^{9} \pm 11$	$104^{9} \pm 4$	$125^{9} \pm 5$	$153^{9} \pm 13$	$150^{9} \pm 14$
$549^{8} \pm 46$	$373^{8} \pm 22$	$423^{9} \pm 16$	$430^{9} \pm 18$	$545^{9} \pm 21$
$222^{9} \pm 10$	$179^{9} \pm 5$	$186^{9} \pm 4$	$215^{9} \pm 9$	$261^{9} \pm 9$
$970^{7} \pm 110$	$680^{9} \pm 20$	$800^{9} \pm 80$	$890^{9} \pm 60$	$1{,}160^{9} \pm 130$
$451^{8} \pm 8$	$477^{8} \pm 17$	$482^{9} \pm 26$	$477^{9} \pm 42$	$526^{9} \pm 27$
$1{,}228^{3} \pm 42$	$1{,}188^{9} \pm 13$	$1{,}267^{9} \pm 26$	$1{,}250^{9} \pm 25$	$1{,}239^{8} \pm 24$
89[9]	89[9]	89[9]	88[9]	89[9]
$263^{9} \pm 11$	$259^{9} \pm 11$	$268^{9} \pm 15$	$342^{9} \pm 10$	$308^{9} \pm 19$
1451	1452	1453	1454	1455
10 1.0	20 0	20 0.1	20 0.3	20 1.0
$2{,}490^{9} \pm 235$	$297^{7} \pm 9$	$322^{9} \pm 11$	$709^{9} \pm 86$	$4{,}585^{9} \pm 498$
$203^{9} \pm 15$	$102^{9} \pm 8$	$141^{9} \pm 10$	$218^{9} \pm 16$	$293^{9} \pm 36$
$1{,}041^{9} \pm 80$	$444^{8} \pm 13$	$460^{9} \pm 9$	$531^{9} \pm 34$	$836^{9} \pm 49$
$431^{8} \pm 44$	$190^{8} \pm 6$	$183^{9} \pm 4$	$245^{9} \pm 15$	$414^{9} \pm 21$
$1{,}870^{9} \pm 170$	$690^{8} \pm 40$	$680^{9} \pm 50$	$950^{9} \pm 90$	$1{,}600^{9} \pm 150$
$651^{9} \pm 33$	$401^{8} \pm 21$	$406^{9} \pm 20$	$467^{9} \pm 20$	$589^{9} \pm 28$
$1{,}166^{9} \pm 20$	$1{,}218^{8} \pm 15$	$1{,}207^{9} \pm 17$	$1{,}213^{9} \pm 21$	$1{,}201^{9} \pm 25$
89[9]	88[9]	88[9]	88[9]	89[9]
$295^{9} \pm 11$	$281^{8} \pm 10$	$264^{9} \pm 18$	$284^{9} \pm 18$	$290^{9} \pm 19$

superscripts. The standard error of the mean is indicated by ±.

not surprising in view of the findings of PAGE and MENSCHICK (1932) with rabbits and STOESSER, PETRI and MCQUARRIE (1935) with rats, who found negligible change of brain cholesterol as a result of cholesterol feeding.

Distribution of polyenoic fatty acids (expressed as percentages of total fatty acids), table 2.

Addition of cholesterol to the diet did not seem to change the distribution of polyenoic fatty acids in the tissues examined. The significance of the relatively low figure found for tetraenoic acid in the liver when 1 % cholesterol and 20 % peanut oil was fed must be evaluated in further studies.

In the *liver*, increasing levels of peanut oil caused increased

Table

Polyenoic acids in % of total fatty acids in tissues of chicks fed diets

Group no.			1440	1441	1442	1443	1444
Additions to diets	% peanut oil		0	0	0	0	3
	% cholesterol		0	0.1	0.3	1.0	0
Liver[1]	% dienoic		2.5	1.3	2.2	1.6	7.9
	% trienoic		3.7	6.9	3.1	3.8	3.5
	% tetraenoic		2.1	3.5	2.2	2.1	7.0
	% pentaenoic		0.3	0.3	0.2	0.5	0.6
	% hexaenoic		0.7	0.5	0.3	0.4	0.9
	% total		9.3	12.5	8.0	8.4	19.9
Heart[2]	% dienoic		6.1	6.2	6.3	6.6	12.6
	% trienoic		7.3	6.7	6.3	6.7	3.4
	% tetraenoic		5.7	5.1	4.5	4.7	9.6
	% pentaenoic		0.4	0.3	0.5	0.4	0.7
	% hexaenoic		0.3	0.3	0.5	0.4	0.5
	% total		19.8	18.6	18.1	18.8	26.8
Lung[3]	% dienoic		1.9	2.4	2.2	0.9	4.0
	% trienoic		4.3	5.0	5.1	4.8	2.6
	% tetraenoic		4.6	6.1	4.3	3.8	6.8
	% pentaenoic		0.6	0.8	0.5	0.3	0.4
	% hexaenoic		0.4	0.6	0.5	0.4	0.4
	% total		11.8	14.9	12.6	10.2	14.2
Brain[2]	% dienoic		—	—	—	—	—
	% trienoic		—	—	—	—	—
	% tetraenoic		—	—	—	—	—
	% pentaenoic		—	—	—	—	—
	% hexaenoic		—	—	—	—	—
	% total		—	—	—	—	—
Fat tissue[2]	% dienoic		1.5	1.5	1.4	1.4	6.9
	% trienoic		0.3	0.3	0.3	0.4	0.4
	% tetraenoic		0.2	0.1	0.2	0.3	0.2
	% pentaenoic		0.1	0.1	0.1	0.1	0.1
	% hexaenoic		0	0.1	0.2	0.2	0.2
	% total		2.1	2.1	2.2	2.4	7.8

[1] The analyses were carried out on individual livers. The figures listed represent
[2] These organs were pooled 4 together for analysis. The results listed are averages
[3] The lungs from 8 animals were pooled for analysis.

deposition of dienoic and tetraenoic acids; however, the difference in deposition of these acids when 10 and 20 % peanut oil was fed was probably insignificant.

Trienoic acid decreased as a consequence of peanut oil feeding, especially when 10 or 20 % was fed.

The increase of the small amounts of penta- and hexaenoic acids present was slight.

2.

with varying amounts of cholesterol and peanut oil for 4 weeks.

1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455
3 0.1	3 0.3	3 1.0	10 0	10 0.1	10 0.3	10 1.0	20 0	20 0.1	20 0.3	20 1.0
8.2	9.0	8.5	11.8	13.8	12.0	12.3	13.6	14.3	15.7	12.5
3.4	3.7	3.2	1.1	1.7	1.4	1.1	1.1	0.9	0.5	0.8
7.0	8.6	6.0	16.8	15.2	11.7	11.4	12.2	12.2	11.7	7.1
0.6	0.8	0.4	2.0	1.1	1.2	1.6	1.4	1.6	1.1	0.5
0.6	0.6	0.6	1.1	1.1	0.9	1.1	1.0	1.3	1.1	0.5
19.8	22.7	18.7	32.8	32.9	27.2	27.5	29.3	30.3	30.1	21.4
12.8	14.8	14.4	13.4	15.5	15.6	15.6	16.5	16.4	17.7	19.0
2.6	3.4	2.7	1.1	0.8	0.8	0.4	0.1	0.2	0	0.4
9.1	11.2	9.8	11.1	11.1	11.5	12.4	14.7	14.2	15.4	12.8
0.8	0.6	0.5	0.8	0.6	0.8	0.8	1.0	1.0	0.9	0.7
0.7	0.2	0.2	0.4	0.3	0.6	0.4	0.5	0.5	0.6	0.4
26.0	30.2	27.6	26.8	28.3	29.3	29.6	32.8	32.3	34.6	33.3
4.2	6.0	5.8	6.1	6.8	6.3	8.3	8.5	9.7	10.0	10.7
1.6	2.5	2.1	1.4	0.5	1.4	1.1	0.8	0.8	0.7	0.5
8.4	8.1	7.7	9.2	8.1	8.3	8.3	10.7	11.3	7.8	9.5
0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.6	0.5	0.5
0.6	0.4	0.4	0.5	0.3	0.5	0.6	0.4	0.4	0.4	0.5
15.4	17.5	16.5	17.8	16.2	17.0	18.8	21.0	22.8	19.4	21.7
—	0.1	0.2	0.6	0.1	0	0	0	0.5	0	0
—	4.5	4.7	2.1	1.5	0.3	0.9	1.0	1.3	0.9	0.6
—	10.7	10.8	12.4	12.9	13.5	12.5	13.3	13.5	11.8	12.1
—	2.2	2.4	3.2	4.1	4.5	3.8	3.7	4.0	3.9	3.8
—	5.0	5.0	4.6	5.9	7.5	6.1	5.4	5.9	5.4	5.2
—	22.5	23.1	22.9	24.5	25.8	23.3	23.4	25.2	22.0	21.7
7.8	6.3	6.4	13.5	12.7	14.1	14.7	17.9	18.2	18.9	18.8
0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.4
0.4	0.3	0.1	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3
0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
0.3	0.2	0.2	0.2	0.3	0.2	0.3	0.1	0.2	0.1	0.2
9.0	7.3	7.2	14.6	13.9	15.1	15.7	18.8	19.2	19.7	19.8

averages for 4 livers.
of two such determinations.

The percentage of total polyenoic fatty acids in the liver increased with increasing dietary peanut oil until the 10 % dietary level. No further increase was obtained by raising the dietary level of peanut oil to 20 %.

In the *heart*, dienoic and tetraenoic acids increased considerably with increasing levels of peanut oil. Trienoic decreased to almost zero at the 20 % peanut oil level. The small amounts

of pentaenoic increased only slightly, and the small amount of hexaenoic was constant. The total amount of polyenoic acids was increased.

In the *lungs*, dienoic and tetraenoic showed marked increases, trienoic a marked decrease, whereas the small amounts of penta- and hexaenoic were constant. The total amount of polyenoic acids was increased.

By an accident the determinations of the *brain* referring to fat-free diets and the diets with 3 % peanut oil + 0 and 0.1 % cholesterol were lost.

From the remaining figures it appears, however, that in the brain peanut oil feeding did not cause increase in dienoic, whereas trienoic decreased. Tetra- and pentaenoic showed slight increase only, and hexaenoic remained constant. The total amount was apparently unchanged.

In the *fat tissue* the only change observed was an increase in dienoic and, as a consequence thereof, also of the sum of polyenoic acids.

None of the animals on the fat-free diets showed external signs of deficiency in essential fatty acids.[1]

The observation that feeding of dienoic fatty acid gives rise to an increase in tetraenoic in certain organs is in agreement with the findings of other authors working with rats (*e. g.* WIDMER and HOLMAN 1950). The decrease of trienoic acid in organ lipids after feeding with dienoic seems to be quite general. A similar effect on trienoic acid in heart lipids after the feeding of corn oil to rats has been found by RIECKEHOFF, HOLMAN and BURR (1949).

The disappearance of trienoic from tissues after feeding of peanut oil might be related to the "sparking effect" of linoleic acid on linolenic, viz. an increase in the utilization of linolenic, described by GREENBERG et al. (1950).

In another study from this laboratory (ENGEL, AAES-JØRGENSEN, FUNCH and DAM, yet unpublished) rats were fed, for 18 weeks from weaning, fat-free diets and such diets with various additions among which was 28 % peanut oil. At the end of the feeding period several tissues were — inter alia — examined for polyenoic

[1] In chicks, signs of deficiency in fat and, probably, in essential fatty acids are among others: edema and "pseudo-encephalomalacia". According to earlier experiences, the incidence of these symptoms is generally low, at least when a few drops of peanut oil a week are given as vehicle for vitamins A and D (DAM, PRANGE and SØNDERGAARD 1952 a and b).

acids. The results of these analyses were largely in agreement with the present findings with chicks.

The average final weight (table 1) of the chicks on fat-free diets and diets with 3 % peanut oil seemed to be higher for 0.3 and 1 % cholesterol than for the corresponding cholesterol-free diets. However, in our previous study (DAM, PRANGE and SØNDERGAARD 1955) addition of cholesterol to the fat-free diet did not show any such influence on the average weight increase in the experimental period. The reason for this discrepancy is unexplained.

Summary.

Cholesterol in liver, fat tissue, spleen, heart, aorta, lungs and brain, and polyenoic fatty acids in liver, heart, lungs, brain and fat tissue were determined in chicks after feeding diets containing 0, 3, 10 or 20 % peanut oil in combination with 0, 0.1, 0.3 or 1.0 % cholesterol for 4 weeks from the 14th day after hatching.

Cholesterol content of the tissues.

Feeding of peanut oil without cholesterol did not result in increase of cholesterol in any of the tissues examined.

The cholesterol values for liver corresponding to 0 and 0.1 % of dietary cholesterol were higher when the diet was fat-free than when it contained 3, 10 or 20 % peanut oil.

The maximal cholesterol deposition obtained under any of the experimental conditions occurred in the liver (with 20 % peanut oil and 1 % cholesterol).

It was possible to produce cholesterol accumulation in fat tissue by combined feeding of cholesterol and peanut oil, but the response was much less than in the liver.

The cholesterol content of spleen, heart, aorta and lungs increased as a consequence of cholesterol and peanut oil feeding, the least response was found in the lungs.

The cholesterol content of the brain showed no change.

Polyenoic fatty acids in the tissues.

Variations of the dietary cholesterol seemed to have no influence, although a relatively low value was found for tetraenoic in the liver for 1 % cholesterol + 20 % peanut oil.

With increasing amounts of dietary peanut oil the following changes were found:

In liver, heart and lungs, dienoic and tetraenoic increased, whereas trienoic decreased. The small amounts of pentaenoic and hexaenoic acids present showed almost no variation.

In brain, dienoic was almost constant and nearly zero, trienoic decreased. Tetra-, penta- and hexaenoic remained constant.

In fat tissue, dienoic increased whereas all the other polyenoic acids were constant and almost zero.

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Alimentary Production of Gallstones in Hamsters. 6. Disappearance of Cholesterol Stones by Treatment with a Non-lithogenic Diet.

By

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In previous communications (DAM and CHRISTENSEN 1952, CHRISTENSEN, DAM and PRANGE 1952, CHRISTENSEN and DAM 1954) we have described the alimentary production of gallstones and the prevention of gallstone formation in hamsters. We have now tried to cure the alimentary cholelithiasis in hamsters by changing the diet from a lithogenic to a non-lithogenic type.

Experimental.

The lithogenic diet was diet no. 295 (CHRISTENSEN, DAM and PRANGE 1952). The non-lithogenic diet (no. 493) contained ground polished rice, Fleischmann yeast type 2019, a small amount of cupric sulfate, a larger amount of lard than diet 295, and no sucrose. The exact composition of the two diets appears from table 1.

Thirty-two hamsters were used for the experiment. They were divided into three groups: A, B and C. Each of groups A and C consisted of 10 animals (5 males and 5 females). Group B consisted of 12 animals (6 males and 6 females). At the beginning of the experiment the hamsters were about 29 days old and weighed about 43 g. All three groups received the lithogenic diet no. 295 for 63 days. Thereafter the animals in group A were killed and autopsied, the gallstones collected, washed in distilled water, dried, weighed, photographed and analyzed for cholesterol by the method of HANEL and DAM (1955). The animals of group B continued on the lithogenic diet for an extra 77 day period, so that when they

were sacrificed they had been on the lithogenic diet for a total of 140 days. The animals of group C were transferred to the non-lithogenic diet on the 64th day of the experiment and received this diet for a 77 day period, at the end of which they were sacrificed.

Autopsy, collection and treatment of gallstones were the same for groups B and C as for group A.

Results and Discussion.

As shown in table 2 all the animals sacrificed after having been on the lithogenic diet for 63 days (group A) had gallstones. Of the animals continuing on the lithogenic diet for the whole experimental period of 140 days (group B) 11 out of 12 had gallstones. Of the animals which were shifted to the non-lithogenic diet after 63 days (group C) 5 out of 10 had gallstones at the end of the experimental period, and the stones in this group were all of the hard, amorphous and dark colored type. The details regarding the characterization of the stones in the three groups appear from the table.

Table 1.

Diet no. 295 (lithogenic).

Casein, crude	20.0 %
Sucrose	72.3 %
Salt mixture (U. S. P. no. 2 XIII)	5.0 %
Vitamin mixture[1]	0.5 %
Choline chloride	0.2 %
Lard with vitamins A and D_3[2]	2.0 %
	100.0 %

Diet no. 493 (non-lithogenic).

Casein, crude	20.0 %
Fleischmann yeast, type 2019	36.0 %
White polished rice (ground)	28.3 %
Salt mixture, U. S. P. no. 2 XIII + 0.13 % $CuSO_4$, $5H_2O$	5.0 %
Vitamin mixture[1]	0.5 %
Choline chloride	0.2 %
Lard	8.0 %
Lard with vitamins A and D_3[2]	2.0 %
	100.0 %

[1] Biotin 50 μg, folic acid 50 μg, ascorbic acid 5 mg, thiamine hydrochloride 5 mg, riboflavin 5 mg, pyridoxine hydrochloride 5 mg, calcium pantothenate 5 mg, nicotinic acid 8 mg, inositol 15 mg, p-aminobenzoic acid 35 mg, vitamin K ("Synkavit" Roche) 1 mg, dl-α-tocopherol acetate ("Ephynal" Roche) 5 mg, and sucrose up to 500 mg.

[2] 2.00 g contained 0.7 mg vitamin A palmitate ("Mywax 16", Distillation Products Industries, Rochester, N. Y.) and 0.667 mg of a solution of irradiated 7-dehydrocholesterol in soybean oil (Ferrosan A/S, Copenhagen), corresponding to 670 I. U. vitamin A and 133 I. U. vitamin D_3.

Table 2.

Gallstones found at autopsy.

Group	Animal no.	Sex	Characterization by aid of dissection and polarization microscope.
A	5	m	Several transparent cholesterol crystals (single crystals and crystal aggregates).
	10	m	3 transparent cholesterol crystal aggregates, 4 yellow-brown rounded amorphous stones, each consisting of 4 or 5 solid units.
	12	m	13 white, opaque cholesterol crystal aggregates. 2 large, white cholesterol stones in ductus cysticus.
	78	m	Several transparent cholesterol crystal aggregates.
	89	m	Several rather large irregular yellow-brown hard facetted stones (mixed type).
	28	f	About 50 small cholesterol stones: a mixture of rounded stones and crystal aggregates.
	59	f	Light yellow rounded crystalline cholesterol stones with crystal edges on the surface, some of them spotted with brown amorphous material which also occurs separately.
	62	f	One large and 3 smaller yellow-white rounded crystalline cholesterol stones and numerous small cholesterol crystals.
	64	f	Single small cholesterol crystals together with yellow-brown amorphous precipitate.
	86	f	Three large and several small rounded crystalline stones, the larger ones being conglomerates of smaller nearly spherical stones. The smaller ones having sharp crystal edges on the surface.
B	17	m	One smaller irregularly shaped yellow-brown amorphous stone with corny surface.
	25	m	No gallstones.
	40	m	One small conglomerate of quite small nearly spherical brown-green amorphous particles.
	24	m	Ten white rounded crystalline cholesterol stones of middle size with some dark-green pigment.
	51	m	Six white crystalline rounded cholesterol stones and some broken stones of same type.
	58	m	Some rather large transparent flat cholesterol crystals grown together and a few yellow-brown small amorphous stones.
	36	f	Six amorphous yellow-green broken stones.
	21	f	About 20 light yellow rounded crystalline cholesterol stones.
	45	f	Many small and large conglomerates consisting of yellow spherical units firmly grown together, spotwise covered by black and green pigment. Some smaller fragments of amorphous stones.
	57	f	Several large rounded crystalline cholesterol stones spotwise with dark-green pigmentation. Small single crystals of rhombic shape.
	70	f	Many whitish rounded, somewhat irregular cholesterol stones.
	84	f	Several rather large amorphous grey-brown to greenish stones with white elongated particles on the surface.
C	18	m	Three very small irregular dark-green amorphous stones.
	26	m	No gallstones.
	27	m	No gallstones.
	37	m	No gallstones.
	48	m	No gallstones.
	34	f	No gallstones.
	38	f	Green and small yellow-green amorphous stones.
	39	f	Some irregular amorphous green stones. Some of them having an easily detatchable green shell.
	79	f	Some small yellow-brown amorphous stones, irregular with respect to color and form.
	83	f	Several very small irregularly shaped amorphous corny stones with a yellow-brown color.

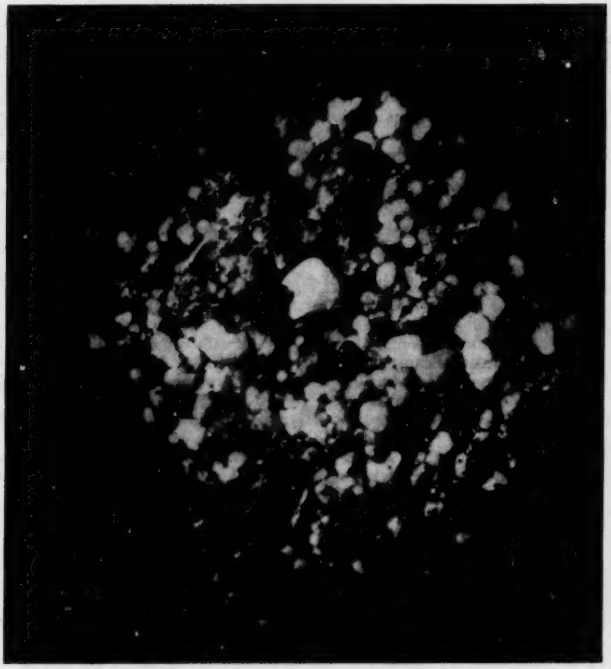

Fig. 1. Gallstones from Group A. (Magnification 10×.)

Table 8.

Weight and cholesterol content of gallstones from each group of hamsters.

Group	No. of animals	Diet		Killed	Total weight of gallstones mg	Cholesterol content of gallstones %
		during first 63 days of experiment	during subsequent 77 days of experiment			
A	10	no. 295 (lithogenic)		at end of first 63 days	9.39	53
B	12	no. 295 (lithogenic)	no. 295 (lithogenic)	at end of subsequent 77 days	10.47	55
C	10	no. 295 (lithogenic)	no. 493 (non-lithogenic)	at end of subsequent 77 days	2.65	< 0.7

Fig. 2. Gallstones from Group B. (Magnification 10×.)

The total weight of stones and the content of cholesterol in the stones in each group are presented in table 3. It is seen that groups A and B have almost the same amount by weight of gallstones (9.39 and 10.47 mg, respectively), and that the percentages of cholesterol in the stones in these groups are almost identical (53 and 55 %, respectively). In the treated group (C), the total weight of stones is much less (2.65 mg), and the percentage of cholesterol in these stones is less than 0.7 %. Therefore, it is evident that the treatment has resulted in the dissolution of cholesterol stones and cholesterol from the amorphous stones.

All the gallstones from each group are shown in figures 1, 2 and 3.

Additional observations in the experiment were that the lithogenic diet no. 295 caused depigmentation of the incisors and

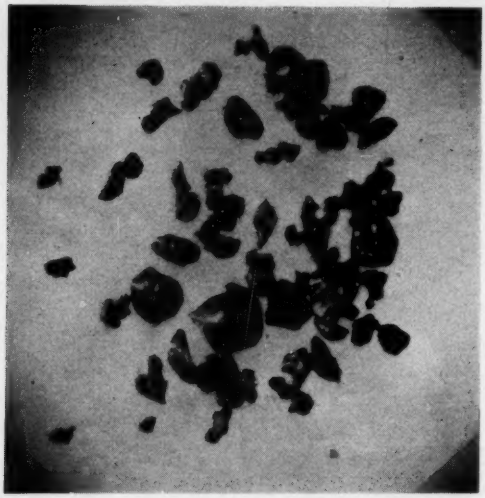

Fig. 3. Gallstones from Group C. (Magnification 10×.)

that the shifting to the non-lithogenic diet caused reappearance of the normal golden-brown color of the incisors. At the end of the experiment the fur color of the animals on the lithogenic diet (groups A and B) was pale and greyish, whereas the fur of the animals in group C which received the non-lithogenic diet for 77 days had the normal red brown color. The fur of these latter animals (receiving 10 % lard in their diet) was somewhat greasy. As shown in table 4 shifting to the non-lithogenic diet caused an improvement of growth. It is not likely, however, that the observed disappearance of cholesterol stones is the cause of the improved growth rate.

Summary.

As shown earlier by us, gallstones consisting of cholesterol and/or pigments can be produced and prevented in hamsters by dietary means.

It has now been shown that cholesterol stones and cholesterol from amorphous stones developed in the gallbladder of hamsters by a lithogenic diet can be dissolved in the bile by changing the diet to a non-lithogenic type.

Table 4.

Average weight (g) of hamsters at weekly intervals.

Group	A		B		C	
Sex	m	f	m	f	m	f
Diet during first 63 days	lithogenic no. 295		lithogenic no. 295		lithogenic no. 295	
Weeks						
0	42	43.4	43.2	43.5	43	41.6
1	48.8	43.6	45.8	47.0	48.8	49.0
2	51.4	46.4	50.7	48.8	51.8	52.2
3	55.4	49.8	54.2	50.3	55.6	52.6
4	56.6	51.6	57.3	51.2	51.4	54.4
5	58.2	53.2	58.3	51.0	58.8	54.8
6	61.0	56.0	60.7	53.0	60.2	57.8
7	63.6	58.5	62.2	55.0	60.4	59.0
8	66.2	61.4	64.3	56.8	60.0	60.6
9	66.6	60.4	64.3	58.2	59.2	59.8
Diet during last 77 days			lithogenic no. 295		non-lithogenic no. 493	
10			64.7	59.2	66.6	63.8
11			64.0	58.7	72.4	73.8
12			63.7	60.0	74.2	78.6
13			63.0	61.2	78.6	83.4
14			64.2	62.5	84.0	87.8
15			62.5	61.5	85.0	89.6
16			60.7	61.2	88.2	91.0
17			59.0	59.0	87.0	90.2
18			62.2	60.8	87.8	94.0
19			64.2	62.3	89.0	97.8
20			64.7	64.7	91.8	100.8

Erratum.

In our paper: Alimentary Production of Gallstones in Hamsters. 3., Acta Physiol. Scand. *31*, 75—82, 1954, page 80, Table 2, Diet 295, footnote 2, between "mg" and "+ sugar" shall be inserted:

", dl-*a*-tocopherol acetate (Ephynal, Roche) 5 mg".

Acknowledgements.

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La Roche & Co., Basle; vitamin A was kindly furnished by Distillation Products Ind., Rochester, N. Y.; vitamin D_3 was kindly furnished by Ferrosan Ltd., Copenhagen.

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A Mathematical Analysis of Indicator Dilution Curves Leading to Direct Calculation of the Systolic and Diastolic Volumes of the Left Ventricle and Auricle and of the Pulmonary Blood Volume.

By

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In 1949 LAGERLÖF, WERKÖ, BUCHT and HOLMGREN described a procedure of calculating the pulmonary blood volume according to the indicator dilution technic of HAMILTON, MOORE, KINSMAN and SPURLING (1932). The total blood volume between the pulmonary artery and the brachial artery was determined. The blood volume in the heart and part of the arterial system was estimated by means of X-ray and subtracted, the difference being the pulmonary blood volume.

The roentgenological estimation of the blood volume in the left heart may be difficult especially in mitral stenosis, when the left auricle is dilated. It is further usually impossible to do simultaneously with the indicator injection procedure. The following paper aims to elucidate the possibilities to calculate the blood volume in the lungs and the left heart directly from the dilution curve, obtained by injecting an indicator in the pulmonary artery and sampling from the brachial artery.

The following preliminary presumptions are made in the calculation.

1. The mean concentration of the indicator ejected during ventricular systole is equal to the mean concentration of the dye at the end of the preceding ventricular diastole. This condition is usually, though not quite correctly, referred to as complete mixing in the left ventricle.

2. The mean concentration of the indicator leaving the left auricle during ventricular diastole is equal to the mean concentration of the indicator at the end of ventricular systole.

3. The indicator dilution curve obtained from the brachial artery is representative of the curve which would have been obtained from the root of the aorta.

4. The pumping mechanism of the heart does not change during the experiment.

$$C_1,\ C_2 \ldots .\ C_n$$

are the indicator concentrations in the first, second etc. systolic ejections of indicated blood.

$$c_1,\ c_2 \ldots .\ c_n$$

are the dye concentrations in the first, second etc. samples filling the left ventricle during the diastole before the first, second etc. systolic ejections of indicated blood.

D = the end diastolic volume of the left ventricle.
S = the stroke volume.

We obtain, C_n being the result of a mixing procedure in the ventricle:

$$C_n = \frac{C_{n-1}\,(D - S) + c_n S}{D} \quad \ldots\ldots\ldots\ldots \quad (1)$$

where $n = 1, 2, 3, \ldots .$ and $C_o = 0$. Putting

$$\frac{D - S}{D} = k, \text{ whence } \frac{S}{D} = 1 - k \quad \ldots\ldots\ldots \quad (2)$$

we write the formula (1), expressing c_n in terms of C_n and C_{n-1}:

$$c_n = \frac{C_n - k\,C_{n-1}}{1 - k} \quad \ldots\ldots\ldots\ldots\ldots \quad (3)$$

As a first attempt, we assume simply that the mixing procedure in the left auricle has an effect analogous to that of the ventricle. Designing by γ_n the indicator concentration ($n = 1, 2, \ldots .$) in the sample filling the left auricle, and by A_s the maximum volume

of the left auricle (in ventricular systole), we get a relation similar to (3):

$$\gamma_n = \frac{c_n - \varkappa c_{n-1}}{1 - \varkappa} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Here, similarly to (2):

$$\varkappa = \frac{A_s - S}{A_s} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

Inserting in (4) the expressions of c_n and c_{n-1} obtained from (3), we get a relation between γ_n on one side and C_n, C_{n-1} and C_{n-2} on the other:

$$\gamma_n = \frac{C_n - (\varkappa + k)\, C_{n-1} + \varkappa k C_{n-2}}{(1 - \varkappa)(1 - k)} \quad \ldots\ldots\ldots \quad (6)$$

After sufficiently long time, *i. e.*, for sufficiently large values of n, say $n \geqq N$, the blood entering the left auricle will be practically empty of indicator. This means that

$$\gamma_n = 0 \text{ for } n \geqq N \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

We do not consider the recirculation; in practice, there will also be an upper limitation for n in (7).

If (7) holds, the relation (6) gives us:

$$C_n - (\varkappa + k)\, C_{n-1} + \varkappa k C_{n-2} = 0 \text{ for } n \geqq N \quad \ldots\ldots \quad (8)$$

Now, it is an experimental fact that the ratios C_n/C_{n-1} end by tending to a constant value. This is most easily seen, if log C_n is plotted against n (*e. g.*, using logarithmic paper). The curve ends in a straight line, if the recirculation is neglected. Let M be the value of n where this straight line begins. Then we have

$$C_{n+1} = K \cdot C_n \text{ for } n \geqq M \quad \ldots\ldots\ldots\ldots\ldots \quad (9)$$

denoting by K the above-mentioned constant ratio. From (9) we obtain

$$C_n = K^{n-M} \cdot C_M \text{ for } n \geqq M \quad \ldots\ldots\ldots\ldots \quad (10)$$

These expressions, based on experimental results, must be in accordance with the relation (8), for such values of n which are simultaneously $\geqq N$ and $\geqq M + 2$. Inserting (10) and the similar expressions of C_{n-1} and C_{n-2} in (8), we get a relation which can be shortened by $K^{n-2-M} \cdot C_M$, thus giving the necessary condition:

$$K^2 - (\varkappa + k)\, K + \varkappa k = 0 \quad \ldots\ldots\ldots\ldots \quad (11)$$

or, in factorized form:

$$(K - \varkappa)(K - k) = 0 \quad \ldots\ldots\ldots\ldots \quad (12)$$

Hence, K necessarily equals either $\varkappa$ or k. If K is calculated by (9) from the measurements, we now know *one* of the quantities $\varkappa$ and k. Because of the complete symmetry it is as yet undecided whether $\varkappa$ or k has the value K.

In order to determine the value of the remaining quantity ($\varkappa$ or k), we calculate the following function:

$$\Gamma_n = \frac{C_n - KC_{n-1}}{1 - K} \text{ for } n = 1, 2, 3, \ldots, M \quad \ldots\ldots\ldots\ldots \quad (13)$$

Let H be the quantity to be determined. Then

$$H + K = \varkappa + k \text{ and } HK = \varkappa k \quad \ldots\ldots\ldots \quad (14)$$

because H and K are the same numbers as $\varkappa$ and k, in some order. So the relation (8) can be written:

$$C_n - (H + K)C_{n-1} + HKC_{n-2} = 0 \text{ for } n \geqq N \quad \ldots\ldots\ldots \quad (15)$$

or, rearranging the terms:

$$(C_n - KC_{n-1}) - H(C_{n-1} - KC_{n-2}) = 0 \text{ for } n \geqq N \quad \ldots\ldots \quad (16)$$

Introducing (13) in (16), we get:

$$\Gamma_n - H\Gamma_{n-1} = 0 \text{ for } N \leqq n \leqq M \quad \ldots\ldots\ldots \quad (17)$$

Without discussing the generality of the property $N \leqq M$, *i. e.* that the indicator concentration of the pulmonary venous blood has decreased to zero before the logarithmic straight line begins which is requested in order to make (17) hold, we find the following observations sufficient for the practical application. We calculate the ratios Γ_n/Γ_{n-1} for those n which correspond to the decreasing Γ_n. If, corresponding to $n = M$, $M - 1$, *etc.*, a number of these ratios are equal, the relation (17) is seen to hold, and the equal ratios give the value of H. In the opposite case, when the ratios Γ_n/Γ_{n-1} are not equal, even not approximately, we are not led to a conclusion, although the ratio Γ_M/Γ_{M-1} might be used as an estimate of the value of H (correct if we knew that $N = M$).

After the determination of H and K, their final identification with $\varkappa$ and k should be based on clinical arguments.

In the preceding deductions, we have assumed that the C_n are measured. This would require the measurements to be made with intervals equal to those between the pulse beats. In practice, the intervals

between the measurements are longer, say, equal to ν pulses. Assuming for the sake of simplicity, that ν is an integer (*e. g.*, $\nu = 2$, 3 or 4), at least approximately, we now show that the deduced method needs only a slight modification.

If C_n is measured, the preceding measurement did not give C_{n-1}, but $C_{n-\nu}$. Therefore, we have to replace (3) and (13) by relations containing C_n and $C_{n-\nu}$ instead of C_n and C_{n-1}. This is easily done by introducing certain mean values $\bar{c}_n$ and $\bar{\Gamma}_n$ which are defined as follows:

$$\bar{c}_n = \frac{c_n + k c_{n-1} + \ldots + k^{\nu-1} c_{n-\nu+1}}{1 + k + \ldots + k^{\nu-1}} \quad \ldots\ldots (18)$$

$$\bar{\Gamma}_n = \frac{\Gamma_n + K\Gamma_{n-1} + \ldots + K^{\nu-1}\Gamma_{n-\nu+1}}{1 + K + \ldots + K^{\nu-1}} \quad \ldots\ldots (19)$$

Inserting (3) in (18), and (13) in (19), we obtain immediately:

$$\bar{c}_n = \frac{C_n - k^\nu C_{n-\nu}}{1 - k^\nu} \quad \ldots\ldots (20)$$

and

$$\bar{\Gamma}_n = \frac{C_n - K^\nu C_{n-\nu}}{1 - K^\nu} \quad \ldots\ldots (21)$$

Concerning (4), we first introduce

$$\bar{\gamma}_n = \frac{\gamma_n + \varkappa\gamma_{n-1} + \ldots + \varkappa^{\nu-1}\gamma_{n-\nu+1}}{1 + \varkappa + \ldots + \varkappa^{\nu-1}}$$

which, with the aid of (4), gives:

$$\bar{\gamma}_n = \frac{c_n - \varkappa^\nu c_{n-\nu}}{1 - \varkappa^\nu} \quad \ldots\ldots (22)$$

Finally, introducing

$$\bar{\bar{\gamma}}_n = \frac{\bar{\gamma}_n + k\bar{\gamma}_{n-1} + \ldots + k^{\nu-1}\bar{\gamma}_{n-\nu+1}}{1 + k + \ldots + k^{\nu-1}} \quad \ldots\ldots (23)$$

we obtain, inserting (22) in (23), and regarding (18):

$$\bar{\bar{\gamma}}_n = \frac{\bar{c}_n - \varkappa^\nu c_{n-\nu}}{1 - \varkappa^\nu} \quad \ldots\ldots (24)$$

The relations (20), (21) and (24) will replace (3), (13) and (4). From (20) and (24) we find that (6) is replaced by

$$\bar{\bar{\gamma}}_n = \frac{C_n - (\varkappa^\nu + k^\nu) C_{n-\nu} + \varkappa^\nu K^\nu C_{n-2\nu}}{(1 - \varkappa^\nu)(1 - k^\nu)} \quad \ldots\ldots (25)$$

This formula gives the mean concentrations, $\bar{\bar{\gamma}}_n$, in the pulmonary vein in terms of C_n, $C_{n-\nu}$, ..., which are the concentrations actually measured with ν pulse beats intervals.

We need not repeat the rest of the deduction, nor shall we give, here, a proof extended to fractional values of ν. The following remark will suffice: Before calculating the volumes by (2) and (5),

one should remember that the preceding calculations have not given k and $\varkappa$, but k^ν and $\varkappa^\nu$.

In order to elucidate the described method, we present a numerical example. As the pulse frequency was 63 and the measurements were made with 2 sec. intervals, the value of ν is 126/60 = 2.1. In the following table, the second column contains the concentration values, measured as many seconds after the injection as indicated in the first column. The subsequent columns have been successively calculated by the described method.

Table I.

Time	C_n	$C_n : C_{n-\nu}$	K^ν	$\bar{I}_n$	$\bar{I}_n : \bar{I}_{n-\nu}$	H^ν	$\bar{\bar{\gamma}}_n$
7	0.090			0.227			0.281
9	1.650			4.020			4.931
11	5.850			12.23			14.19
13	7.250			9.38	0.767		8.65
15	5.700	0.786		3.345	0.357		1.881
17	3.700	0.649		0.662	0.198	0.183	0.031
19	2.275	0.615	0.603	0.111	0.168		(— 0.002)
21	1.340	0.589		(— 0.081)			
23	0.810	0.605		(+ 0.005)			
25	0.535	0.661		(+ 0.118)			

Finally, as $\nu = 2.1$, we obtain:

$$K = (0.603)^{1/2.1} = 0.786$$

$$H = (0.183)^{1/2.1} = 0.445$$

which are the values of k and $\varkappa$, in some order. The cardiac output per minute being 3,940 cm³, we get

$$S = \frac{3{,}940}{63} = 62.5 \text{ cm}^3$$

The relations (2) and (5) give us:

$$D = \frac{S}{1 - k} \text{ and } A_s = \frac{S}{1 - \varkappa}$$

Employing the calculated values of K, H and S, we obtain:

$$\frac{S}{1 - K} = \frac{62.5}{0.214} = 292 \text{ cm}^3, \text{ and}$$

$$\frac{S}{1-H} = \frac{62.5}{0.555} = 113 \text{ cm}^3$$

being the volumes D and A_s, in some order.

If the γ values are plotted in a coordinate system with the concentration on the ordinate and the time on the abscissa we obtain the appearance of the indicator curve at the entrance of the left auricle. From this curve or directly from the obtained figures the mean circulation time of the lungs will be obtained according to the formula

$$T_m = \frac{\bar{\gamma}_1 t_1 + \bar{\gamma}_2 t_2 + \ldots + \bar{\gamma}_n t_n}{\bar{\gamma}_1 + \bar{\gamma}_2 + \ldots + \bar{\gamma}_n} - t$$

where t_n is the time from the moment of injection to that of the measuring of C_n, while t is the (constant) time difference between those moments to which C_n, respectively $\bar{\gamma}_n$ refer. In fact, the notations C_n and $\bar{\gamma}_n$ are chosen so that, if C_n refers to the end of the nth diastole, then $\bar{\gamma}_n$ can be considered as a mean value for the interval between the (n — 2)nd and the (n — 1)st diastole. Because a more exact value of t is not necessary here, we assume it to be the time for 1.5 pulse beats.

The blood volume of the lungs V is then

$$V = \frac{T_m \cdot C_o}{60}$$

where C_o is the cardiac output (HAMILTON et al. 1932). The blood volume of the lungs may also be calculated as the difference between the total cardiopulmonary blood volume calculated from the original curve and the average blood volume in the left heart *i. e.*

$$\frac{2A_s - S}{2} + \frac{2D - S}{2}$$

where A_s as before is the maximum volume of the auricle, D the end diastolic volume of the left ventricle and S the stroke volume.

In our numerical example, we take the values of t_n, C_n and $\bar{\gamma}_n$ from table I. At the calculation of the total cardiopulmonary blood volume, the sums should be extended to $n = \infty$. Thus, the terms for which C_{n+1}/C_n is constant give the sums:

$$C_n + C_{n+1} + \ldots = C_n (1 + K^\nu + K^{2\nu} + \ldots) = \frac{C_n}{1 - K^\nu}$$

$$t_n C_n + t_{n+1} C_{n+1} + \ldots = t_n \cdot \frac{C_n}{1 - K^\nu} + (t_{n+1} - t_n) \cdot \frac{C_n K^\nu}{(1 - K^\nu)^2}$$

These are called "end sums" in the following table.

Table II.

t_n	C_n	$t_n C_n$	$\bar{\gamma}_n$	$t_n \bar{\gamma}_n$
7	0.090	0.63	0.281	1.97
9	1.650	14.85	4.931	44.38
11	5.850	64.35	14.19	156.09
13	7.250	94.25	8.65	112.45
15	5.700	85.50	1.881	28.22
17	—	—	0.031	0.53
End sums	9.320	186.75	—	—
Sums	29.86	446.33	29.96	343.64

Thus, the mean circulation time of the lungs and the left heart is:

$$\frac{446.33}{29.86} = 14.95 \text{ sec.}$$

while, as

$$t = 1.5 \cdot \frac{60}{63} = 1.43 \text{ sec.}$$

that of the lungs will be:

$$\frac{343.64}{29.96} - 1.43 = 10.04 \text{ sec.}$$

The corresponding blood volumes are:

$$14.95 \cdot \frac{3{,}940}{60} = 982 \text{ cm}^3$$

and

$$10.04 \cdot \frac{3{,}940}{60} = 660 \text{ cm}^3$$

Their difference, 322 cm³, has to agree with the mean volume of the left heart. As formerly mentioned, this could be calculated as $A_s + D - S$. Using the values obtained earlier, we get

$$A_s + D - S = 292 + 113 - 62{,}5 = 342{,}5 \text{ cm}^3$$

Thus, the values are in good accordance to each other.

Comments.

The validity of the relationship: volume = flow times mean circulation time proposed by HAMILTON, MOORE, KINSMAN and SPURLING 1932 has recently been elucidated by MEIER and ZIERLER (1954). It gives the possibility to calculate the blood volume between the point of indicator injection in the pulmonary artery and any place downstream, where the indicator concentration at the first circulation can be followed or calculated. As shown by LAGERLÖF, WERKÖ, BUCHT and HOLMGREN 1949, a necessary presumption for this is either that the dye is evenly mixed with blood before the branching of the right or left pulmonary artery or that the relation between pulmonary blood volume and flow are equal in both lungs.

One of the scopes of the present method has been to construct the indicator dilution curve, as it appears in the pulmonary veins. By means of this curve the pulmonary blood volume can be calculated directly. In this calculation it is presumed that the concentrations in the brachial artery are identical with those in the root of aorta, further that the equal concentrations appear at the same time. Evidently this approximation gives a somewhat too high mean circulation time for the cardiopulmonary and pulmonary circulation and consequently for the corresponding blood volumes. The obtained mean circulation time should be shortened by the mean circulation time between the root of the aorta and the brachial artery, Tm_{ao}. If the resistance in different parts of the body were equal and if the total cross section of the proximal artery does not change with branching this figure should be V_{no}/Co, where V_{ao} is the volume of aorta and all its branches to a distance equal to the site of the sampling place in the brachial artery. As discussed before (LAGERLÖF, WERKÖ, BUCHT and HOLMGREN 1949), V_{no} lies around 250 ml. It is evident, that a free flow from an indwelling arterial needle and warming of the arm are measures, that will shorten Tm_{ao} and decrease this systematic error.

The given analysis of the appearance of the indicator dilution curve differs from those of NEWMAN, MERRELL, GENECIN, MONGE, MILNOR and MC KEEVER (1951), STEPHENSON (1948) and SHEPPARD (1954). These do not seem to have led to acceptable practical results. It is presumed, that not only the ventricles, but also the auricles empty discontinuously. It is further presumed that blood emptied from the ventricle

has the same mean concentration as the blood contained in the ventricle at the end of the diastole and that the blood emptied from the auricle has the same concentration as the blood contained in the auricle at the end of systole. In the ventricle this condition must be fulfilled, because no blood enters it during systole. In the auricle the situation is more complicated. At the end of systole it is filled with blood. During diastole it delivers the stroke volume to the left ventricle. Probably the largest part (Q) of this volume is emptied during the rapid filling phase and hence has approximately the same mean concentration as the mixed end-systolic volume. The pressure fall in the p.c.v. curve corresponding to this phase indicates, that at least some of this blood may be replaced by pulmonary venous blood which will be able to enter the ventricle especially during auricular systole.

Further, mitral and aortic regurgitation may appear. During diastole, a volume (Z) enters the ventricle from the aorta, whereas during systole, a volume (R) comes to the auricle from the ventricle.

All these phenomena will affect our formulae more or less deeply. Formally, the relation (3) remains unaltered, but k will no longer be defined as in (2). In (4), an additional term will appear, depending on C_{n-1}, and $\varkappa$ is an other quantity than in (5). However, in the fundamental relation (6), only the coefficients are changed. The method of treatment indicated above in the uncomplicated case will, therefore, be the same in the more general case. It is the subject of an additional paper to show to what extent the quantities, Q, R and Z can be determined, or estimated, by this method.

Summary.

A mathematical analysis of indicator dilution curves obtained by indicator injection into the pulmonary artery and blood sampling from the brachial artery is given. By considering the dilution effect of the left ventricle and auricle successively, the appearance of the dye dilution curve at the entrance of the left auricle is obtained. By means of this curve the mean circulation time of the lung is obtained and used for the calculation of the pulmonary blood volume.

The diastolic volume of the left ventricle and the systolic of the left auricle are obtained from the appearance of the last part of the curve, considering the dilution effect of each.

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An Extended Analysis of Indicator Dilution Curves, Leading to Direct Calculation of the Pulmonary Blood Volume and the Blood Volume in the Left Heart even in the Presence of Aortic and Mitral Regurgitation.

By

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In an earlier paper (COMÉT and LAGERLÖF 1956) a new mathematical method of analysing indicator dilution curves obtained by indicator injection into the pulmonary artery and blood sampling from the brachial artery was given. By considering the dilution effect of the left ventricle and auricle successively, the appearance of the dye dilution curve at the entrance of the left auricle is obtained.

By means of this curve the mean circulation time of the lung is obtained and used for the calculation of the pulmonary blood volume.

The diastolic volume of the left ventricle and the systolic of the left auricle are calculated from the appearance of the last part of the curve, considering the dilution effect of each.

In our previous paper (COMÉT and LAGERLÖF 1956), it was assumed that the result of the mixing procedure in the left auricle can be described by a recursion formula analogue to the one corresponding to the left ventricle. It was further assumed that no regurgitation occurred in the mitral or aortic valve.

It is possible to replace these assumptions by a more detailed examination of the changes in the indicator concentrations during one pulse beat, including the effect of regurgitation.

We denote by V_s and D respectively the end-systolic and the end-diastolic volumes of the left ventricle, and by A_1 and A_D respectively the maximum and the minimum volumes of the left auricle. The ventricular *diastole* will be considered as consisting of two periods. During the *first period*, a blood volume = Q enters the ventricle from the auricle, which is, simultaneously, receiving the same amount of blood from the vein; thus, the volume of the auricle is supposed to be constant ($= A_s$) during this period. By regurgitation, a blood volume = Z_1 enters the ventricle from aorta. During the *second period* of ventricular diastole, the auricle contracts, delivering a blood volume = $A_s - A_D$ to the ventricle, which also receives a regurgitated volume = Z_2 from aorta. The total regurgitated volume from aorta is called Z, thus

$$Z = Z_1 + Z_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Finally, during ventricular *systole*, the ventricle delivers a blood volume = S to aorta and regurgitates a volume = R to the auricle. The simultaneous increase of the volume of the auricle, from A_D to A_s, causes the entering of a blood volume = $A_s - A_D - R$ from the vein.

For clearness, we give, in the following scheme, the direction ("dir.") of the blood flow, the volume ("vol.") of the transported blood, and the indicator concentration ("ind. conc."), during each of the mentioned three periods of the nth pulse beat. We indicate also some volumes and indicator concentrations in the end phases of the periods.

We assume that the indicator concentrations can be considered as constants during each of the periods, *viz.* equal to a mean value corresponding to the period in question.

The resulting blood flow through the left heart during one complete pulse beat is called V, and we have obviously, attention payed to (1):

$$V = S - Z = Q + A_s - A_D - R \quad \ldots\ldots\ldots \quad (2)$$

For convenience, we introduce the total blood volume, U, delivered from the auricle to the ventricle during diastole. Observing (2), we get

$$U = Q + A_s - A_D = V + R = S + R - Z \quad \ldots\ldots \quad (3)$$

		vein	auricle	ventricle	aorta
End of $(n-1)$st systole	vol.		A_s	V_s	
	ind. conc.	γ_{m_n}	c_{s_n}	C_{n-1}	C_{n-1}
1st period of nth diastole	dir.		$\rightarrow$	$\rightarrow$	$\leftarrow$
	vol.		Q	Q	Z_1
	ind. conc.		γ_{m_n}	c_{s_n}	C_{n-1}
End of 1st period	vol.		A_s		
	ind. conc.		c_{A_n}		C_{n-1}
2nd period of nth diastole	dir.			$\rightarrow$	$\leftarrow$
	vol.			$A_s - A_D$	Z_2
	ind. conc.			c_{A_n}	C_{n-1}
End of nth diastole	vol.		A_D	D	
	ind. conc.	γ_{s_n}	c_{A_n}	C_n	C_{n-1}
nth systole	dir.		$\rightarrow$	$\leftarrow$	$\rightarrow$
	vol.		$A_s - A_D - R$	R	S
	ind. conc.		γ_{s_n}	C_n	C_n
End of nth systole	vol.		A_s	V_s	
	ind. conc.	$\gamma_{m_{n+1}}$	$c_{s_{n+1}}$	C_n	C_n

The ventricle volumes V_s and D are connected by the relation:

$$D = V_s + S + R \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

We obtain three equations in considering how the amount of indicator contained in a cavity (*viz.* the ventricle or the auricle) varies during a certain time interval. Thus, for the ventricle, during the nth diastole (*cf.* the scheme above):

$$C_n D = C_{n-1} V_s + C_{n-1} Z + c_{s_n} Q + c_{A_n} (A_s - A_D) \quad \ldots \quad (5)$$

and for the auricle, during the first period of the nth diastole:

$$c_{A_n} A_s = c_{s_n} A_s - c_{s_n} Q + \gamma_{m_n} Q \quad \ldots\ldots\ldots\ldots \quad (6)$$

and during the nth systole:

$$c_{s_{n+1}} A_s = c_{A_n} A_D + \gamma_{s_n} (A_s - A_D - R) + C_n R \quad \ldots\ldots \quad (7)$$

The relation (5) is more detailed than the corresponding one of our previous paper [I, relation (1)][1]. We define a mean indicator concentration, c_n, in the auricle during the nth diastole putting

$$c_n (Q + A_s - A_D) = c_{s_n} Q + c_{A_n} (A_s - A_D) \quad \ldots\ldots (8)$$

Using (3), (4), and (8), we can write (5) in the form:

$$C_n D = C_{n-1} (D - U) + c_n U \quad \ldots\ldots\ldots\ldots (9)$$

In the absence of regurgitations, *i. e.* $R = Z = O$, it gives the relation [I, (1)], as easily seen.

Here, and in the following calculations, it is convenient to introduce the ratios of the volumes with respect to the volume U. Thus, we write at once all the needed ratios:

$$\frac{D}{U} = d; \quad \frac{A_s}{U} = a; \quad \frac{Q}{U} = q; \quad \frac{R}{U} = r; \quad \frac{Z}{U} = z; \quad \ldots\ldots (10)$$

and observe that, by (3) and (10):

$$\frac{S}{U} = 1 - r + z \quad \ldots\ldots\ldots\ldots\ldots (11)$$

$$\frac{A_D}{U} = q + a - 1 \quad \ldots\ldots\ldots\ldots\ldots (12)$$

and

$$\frac{V}{U} = 1 - r \quad \ldots\ldots\ldots\ldots\ldots\ldots (13)$$

Introducing (10) into (9) we obtain

$$C_n = C_{n-1}\left(1 - \frac{1}{d}\right) + c_n \cdot \frac{1}{d} \quad \ldots\ldots\ldots\ldots (14)$$

It is possible to eliminate all c_{A_n} and c_{s_n} from the relations (6), (7), and (8). First, we rewrite them with regard to (10) and (12):

$$c_{A_n} a = c_{s_n} a - c_{s_n} q + \gamma_{m_n} q \quad \ldots\ldots\ldots\ldots (15)$$

$$c_{s_{n+1}} a = c_{A_n} (q + a - 1) + \gamma_{s_n} (1 - q - r) + C_n r \ldots (16)$$

$$c_n = c_{s_n} q + c_{A_n} (1 - q) \quad \ldots\ldots\ldots\ldots\ldots (17)$$

[1] The notation I refers to our previous paper.

The elimination can be performed as follows. The sum of the left members of (15) and (17) being equal to the sum of the right members, we get

$$c_{A_n}(a + q - 1) = c_{s_n} a - c_n + \gamma_{m_n} q$$

which, inserted in (16), gives:

$$c_{s_{n+1}} a = c_{s_n} a - c_n + \gamma_{m_n} q + \gamma_{s_n}(1 - q - r) + C_n r \quad \ldots\ldots (18)$$

Multiplying (15) by $(1 - q)$ and (17) by a, we get:

$$c_n a = c_{s_n} a - c_{s_n} q (1 - q) + \gamma_{m_n} q (1 - q)$$

or

$$c_{s_n}[a - q(1 - q)] = c_n a - \gamma_{m_n} q (1 - q) \quad \ldots\ldots (19)$$

We multiply all the terms of (18) by the factor $[a - q(1 - q)]$ and insert the expression (19) and its analogue with n changed to $n + 1$. Thus, we obtain

$$\begin{aligned}[c_{s_{n+1}} a - \gamma_{m_{n+1}} q (1 - q)]\, a = {} & [c_n a - \gamma_{m_n} q (1 - q)]\, a - \\ & - c_n [a - q(1 - q)] + C_n r [a - q(1 - q)] + \\ & + [\gamma_{m_n} q + \gamma_{s_n}(1 - q - r)][a - q(1 - q)]\end{aligned}$$

Dividing all the terms by a^2 and putting

$$q \frac{(1 - q)}{a} = f \quad \ldots\ldots\ldots\ldots\ldots (20)$$

we obtain:

$$\begin{aligned}c_{n+1} = {} & c_n \left(1 - \frac{1 - f}{a}\right) + C_n \frac{r(1 - f)}{a} + (\gamma_{m_{n+1}} - \gamma_{m_n}) f + \\ & + [\gamma_{m_n} q + \gamma_{s_n}(1 - q - r)] \cdot \frac{1 - f}{a} \quad \ldots\ldots\ldots (21)\end{aligned}$$

We introduce the following mean value, γ_{n+1}, of the indicator concentration in the pulmonary vein:

$$\begin{aligned}\gamma_{s_{n+1}} (1 - r)(1 - f) = {} & (\gamma_{m_{n+1}} - \gamma_{m_n}) f a + \\ & + [\gamma_{m_n} q + \gamma_{s_n}(1 - q - r)](1 - f) \quad \ldots\ldots\ldots (22)\end{aligned}$$

Then, the relation (21) becomes, if n in the subscripts is changed to $n - 1$:

$$c_n = c_{n-1} \left(1 - \frac{1 - f}{a}\right) + C_{n-1} \frac{r(1 - f)}{a} + \gamma_n \frac{(1 - r)(1 - f)}{a} \quad (23)$$

This relation corresponds to [I, (4)]. Together with (14), it relates the indicator concentrations, γ_n, in the pulmonary vein with those, C_n, in aorta.

The coefficient of C_{n-1} in (14) and that of c_{n-1} in (23) are of importance; we call them k and h:

$$k = 1 - \frac{1}{d} \quad \ldots\ldots\ldots\ldots\ldots\ldots (24)$$

$$h = 1 - \frac{1-f}{a} = 1 - \frac{1}{a} + \frac{q(1-q)}{a^2} \quad \ldots\ldots (25)$$

In these notations, (14) is written

$$C_n = kC_{n-1} + (1-k)\, c_n$$

or, solved for c_n:

$$c_n = \frac{C_n - kC_{n-1}}{1-k} \quad \ldots\ldots\ldots\ldots\ldots (26)$$

and (23):

$$c_n = hc_{n-1} + r(1-h)\, C_{n-1} + (1-r)(1-h)\, \gamma_n$$

or, solved for γ_n

$$\gamma_n = \frac{c_n - hc_{n-1} - r(1-h)\, C_{n-1}}{(1-r)(1-h)} \quad \ldots\ldots\ldots (27)$$

Here, we insert the expressions for c_n and c_{n-1} which are given by (26). The resulting formula

$$\gamma_n = \frac{C_n - [h + k + r(1-h)(1-k)]\, C_{n-1} + hkC_{n-2}}{(1-r)(1-h)(1-k)} \quad \ldots (28)$$

expresses the mean concentrations γ_n in the pulmonary vein in terms of the concentrations in aorta which are accessible to experimental determination. We shall now show how to obtain the coefficients in this formula.

Repeating the considerations of our previous paper, we note that the pulmonary vein is practically empty of indicator when sufficiently long time has passed since the injection (we assume that the recirculation has not yet begun to influence the indicator concentration in the vein). This means that an N exists such that

$$\gamma_n = 0 \text{ for } n \geqq N \quad \ldots\ldots\ldots\ldots\ldots (29)$$

It follows from (28) and (29) that

$$C_n - [h + k + r(1 - h)(1 - k)]\, C_{n-1} + hk\, C_{n-2} = 0 \quad \ldots\ (30)$$

for $n \geqq N$. The general solution C_n, as a function of the integer-valued variable n ($= N,\ N + 1,\ N + 2,\ \ldots$), is obtained as follows, according to the theory of recurrent series.

Let $x = H$ and $x = K$ be the roots of the 2nd degree equation

$$x^2 - [h + k + r(1 - h)(1 - k)]\, x + hk = 0 \quad \ldots\ (31)$$

Then,

$$C_n = P_1 H^n + P_2 K^n, \text{ if } H \neq K$$

$$C_n = (P_3 n + P_4)\, K^n, \text{ if } H = K,$$

where P_1 and P_2, respectively P_3 and P_4, are expressible by means of the first two terms of the series, *i. e.* in our case by means of C_N and C_{N+1}; the nearer details are of no importance to our discussion.

In the case $H \neq K$, we conclude:

if $P_1 = 0$: $\quad C_{n+1}/C_n = K$;

if $P_2 = 0$: $\quad C_{n+1}/C_n = H$;

if $P_1 \neq 0,\ P_2 \neq 0,\ H < K$: $\quad C_{n+1}/C_n = K\left(P_1\left(\frac{H}{K}\right)^{n+1} + P_2\right) / \left(P_1\left(\frac{H}{K}\right)^{n} + P_2\right)$

which approaches $C_{n+1}/C_n = K$, as n increases; if $P_1 \neq 0$, $P_2 \neq 0$, $H > K$, we deduce similarly that C_{n+1}/C_n approaches H, as n increases.

In the case $H = K$:

$$C_{n+1}/C_n = K\,(P_3 n + P_3 + P_4)/(P_3 n + P_4)$$

which approaches $C_{n+1}/C_n = K$, as n increases.

Therefore, one of the roots is obtained as the limit approached by C_{n+1}/C_n for increasing n. We specify the notation K for the root so determined. Before deducing the method of determining the remaining root H, we remark that, H and K being the roots of the equation (31), we have

$$\left.\begin{aligned} H + K &= h + k + r(1 - h)(1 - k), \\ HK &= hk \end{aligned}\right\} \quad \ldots\ (32)$$

Consequently, the recurrence relation (30) can be written

$$C_n - (H + K)\, C_{n-1} + HK C_{n-2} = 0 \text{ for } n \geqq N \quad .. (33)$$

As in our previous paper, we calculate the quantities

$$\Gamma_n = \frac{C_n - K C_{n-1}}{1 - K} \quad \ldots\ldots\ldots\ldots (34)$$

Inserting them into (33), we get:

$$\Gamma_n - H\Gamma_{n-1} = 0 \text{ for } n \geqq N \quad \ldots\ldots\ldots (35)$$

showing that H is obtained as the final constant value of Γ_n/Γ_{n-1} (*cf.* paper I).

After finding the values of H and K, we can calculate γ_n according to (28). First, we form, using the relations (32):

$$\begin{aligned}(1-H)(1-K) &= 1 - H - K + HK = \\ &= 1 - h - k - r(1-h)(1-k) + hk = \\ &= (1-h)(1-k) - r(1-h)(1-k) = \\ &= (1-r)(1-h)(1-k) \ldots\ldots\ldots\ldots (36)\end{aligned}$$

which is the denominator of (28). Introducing (32) and (36) into (28), we obtain

$$\gamma_n = \frac{C_n - (H + K)\, C_{n-1} + HK C_{n-2}}{(1-H)(1-K)} \quad \ldots\ldots (37)$$

It is now possible to calculate the mean circulation times (*cf.* paper I):

$$T_m = \frac{t_1 C_1 + t_2 C_2 + \ldots.}{C_1 + C_2 + \ldots.} \quad \ldots\ldots\ldots\ldots (38)$$

and

$$T'_m = \frac{t_1\gamma_1 + t_2\gamma_2 + \ldots.}{\gamma_1 + \gamma_2 + \ldots.} - t \quad \ldots\ldots\ldots (39)$$

where t_1, t_2, ... are the moments (counted from the moment of injection) at which the concentrations C_1, C_2, ..., respectively, are ejected in aorta. By the definition (22) of γ_{n+1} as a mean value of the concentration in the vein during the interval between t_{n-1} and t_n, it is clear that t has a value between the time for one and that for two pulse beats. Let τ be the time for each pulse beat. Then

$$t_n = t_1 + (n-1)\,\tau \quad \ldots\ldots\ldots\ldots\ldots (40)$$

and

$$t = \lambda\tau \text{ with } 1 < \lambda < 2 \quad \ldots\ldots\ldots\ldots (41)$$

Inserting (37) in (39), we obtain

$$T'_m + t = \frac{\Sigma t_i C_i - (H + K)\,\Sigma t_i C_{i-1} + HK\Sigma t_i C_{i-2}}{\Sigma C_i - (H + K)\,\Sigma C_{i-1} + HK\Sigma C_{i-2}} =$$

$$= \frac{(1 - H)(1 - K)\,\Sigma t_i C_i - (H + K)\,\tau\Sigma C_i + HK \cdot 2\tau\Sigma C_i}{(1 - H)(1 - K)\,\Sigma C_i}$$

which gives, with regard to (38):

$$T'_m + t = T_m - \frac{H + K - 2HK}{(1 - H)(1 - K)} \cdot \tau$$

or, using (41):

$$T'_m = T_m - \left(\frac{1 - HK}{(1 - H)(1 - K)} - 1 + \lambda \right) \tau \quad \ldots\ldots (42)$$

This is a simpler expression for T'_m than (39).

We are interested in the pulmonary blood volume, which is obtained as $= \frac{T'_m \cdot V}{\tau}$. Here, V is the known volume defined by (2). The only unknown quantity in the expression is λ. In our previous paper, we suggested to put $\lambda = 1.5$ as a reasonable approximation. We will examine this point in more detail in the appendix.

The mean volume, W, of the left heart is given by

$$W = (T_m - T'_m) \cdot \frac{V}{\tau} \quad \ldots\ldots\ldots\ldots (43)$$

that is, by means of (42):

$$W = \left(\frac{1 - HK}{(1 - H)(1 - K)} - 1 + \lambda \right) \cdot V \quad \ldots\ldots\ldots (44)$$

Discussion.

The more complete analysis of the mixing conditions in the left auricle, given in this paper, shows that the approximate assumption of similar mixing conditions in the left auricle as in the ventricle used in our preceding paper does not introduce any error of practical significance. It further shows, that even in the presence of aortic or mitral regurgitation — or both — the indicator concentration in the pulmonary vein can be calculated according to the method used in the previous paper. From this

concentration the pulmonary mean circulation time and the pulmonary blood volume may be derived. This may be done by the formula of HAMILTON, MOORE, KINSMAN and SPURLING (1932) or according to a simpler expression given in (42). The difference between the total blood volume between the pulmonary artery and the brachial artery on one side and between the pulmonary artery and the pulmonary veins on the other side gives the mean blood volume of the left heart.

By means of rather complicated mathematical evaluations the volume of the left heart may also be estimated directly. The analysis elucidates the significance of the empirical constants H and K, which determine the slope of the last part of the dilution curve.

Summary.

An extended mathematical analysis of indicator dilution curves obtained by injection of indicator into the pulmonary artery and blood sampling from the brachial artery is given.

It is shown that the previously given method to obtain the pulmonary blood volume holds even under the complicated conditions of regurgitation from the mitral or aortic valve or both. Under these conditions the volume of the left heart can still be determined though it is impossible to separate the volume of the auricle and ventricle.

Appendix.

This volume of the left heart can also be estimated in a different way. To do this, we consider one pulse beat, of duration τ, as divided into the three periods specified in the beginning of this paper. Let the durations of these periods be denoted by τ_1, τ_2, and τ_3, respectively. Assuming that, within each of the periods, the *sum* of the volumes of the left auricle and the left ventricle changes proportionally to time, the mean value, W, of this sum may be estimated by

$$W = A_s + V_s + \frac{(Q + Z_1)\tau_1 + (2Q + 2Z_1 + Z_2)\tau_2 + (Q + Z_1 + Z_2)\tau_3}{2(\tau_1 + \tau_2 + \tau_3)}$$

According to (1), this can be written:

$$W = A_s + V_s + \frac{Q + Z}{2} + \frac{Q\tau_2 + Z_1\tau_2 - Z_2\tau_1}{2(\tau_1 + \tau_2 + \tau_3)} \quad \ldots\ldots (45)$$

Here, we will delete the terms $Z_1\tau_2 - Z_2\tau_1$, because their contribution to the value of (45) is comparatively small. Inserting (4), (10), and (11) in (45), and putting

$$\frac{\tau_2}{\tau} = \varepsilon, \ (\tau_1 + \tau_2 + \tau_3 = \tau) \quad \ldots\ldots\ldots\ldots \quad (46)$$

we get:

$$W = \left(a + d - 1 - z + \frac{q + z}{2} + \frac{q\varepsilon}{2}\right) \cdot U \quad \ldots\ldots\ldots \quad (47)$$

Now, we have from (24):

$$d = \frac{1}{1 - k} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (48)$$

and from (25):

$$a = \frac{1 + \sqrt{1 - 4q\,(1-q)\,(1-h)}}{2\,(1-h)} \quad \ldots\ldots\ldots\ldots \quad (49)$$

the square root being taken with its positive sign, because a must be > 1. Introducing the quantity

$$\delta = \frac{1 - \sqrt{1 - 4q\,(1-q)\,(1-h)}}{2\,(1-h)} \quad \ldots\ldots\ldots\ldots \quad (50)$$

we write (49):

$$a = \frac{1}{1-h} - \delta \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (51)$$

With (48) and (51) inserted, the expression (47) becomes

$$W = \left(\frac{1 - hk}{(1-h)\,(1-k)} - \delta + \frac{q - z + q\varepsilon}{2}\right) \cdot U$$

or with regard to (13), (32) and (36):

$$W = \left(\frac{1 - HK}{(1-H)\,(1-K)} - \frac{\delta}{1-r} + \frac{q - z + q\varepsilon}{2\,(1-r)}\right) \cdot V \quad \ldots \quad (52)$$

A comparison of the expressions (44) and (52) gives the relation:

$$\lambda - 1 = \frac{q\,(1+\varepsilon) - z - 2\delta}{2\,(1-r)} \quad \ldots\ldots\ldots\ldots \quad (53)$$

For a heart without regurgitation ($r = z = 0$) we get

$$\lambda = 1 + \frac{q\,(1+\varepsilon) - 2\delta}{2}$$

In reality, the value of q (the part of the stroke volume which enters the ventricle during auricular diastole) lies between $^1/_2$ and 1. For $q = {}^1/_2$, δ attains its maximum value, according to (50),

$$\delta_{max} = \frac{1}{2\,(1 + \sqrt{h})}$$

and

$$\lambda = 1 + \frac{1 + \varepsilon}{4} - \frac{1}{2\,(1 + \sqrt{h})}$$

For $q = 1$, we have $\delta = 0$ and

$$\lambda = 1 + \frac{1+\varepsilon}{2}$$

Therefore, we see that the value $\lambda = 1^1/_2$, proposed in our previous paper, is suitable for use in (42) and (44) for a heart without regurgitation, the error introduced by this approximation being of minor importance.

In the presence of regurgitation, however, the expression (53) shows how λ is to be modified. At the calculation of δ according to (50), one needs the value of h. This could be obtained from the system (32), though not uniquely, observing that from (32) (and (36)) it follows:

$$\left.\begin{aligned} h + k &= H + K - \frac{r}{1-r}(1-H)(1-K) \\ hk &= HK \end{aligned}\right\}$$

so that h and k are the roots of the equation in the unknown x:

$$x^2 - \left[H + K - \frac{r}{1-r}(1-H)(1-K)\right]x + HK = 0 \quad .. (54)$$

Graphically represented, these roots are the abscissas of the intersections between the parabole

$$y = x^2 - (H+K)x + HK$$

and the straight line

$$y = -\frac{r}{1-r}(1-H)(1-K)x$$

If $r = 0$, the roots h and k are equal to H and K in some order. If $r > 0$, the roots h and k lie, both of them, between H and K. This makes it possible to estimate the value of δ. In order to make a distinction which of the roots is h, one will have to compare the values of d and a calculated from (48) and (51) with a clinical observation of the relative sizes of the ventricle and auricle. At this comparison, one could use the numbers H and K as approximate values of the roots (exact if $r = 0$!).

The value of ε, defined by (46), could be measured from an electrocardiogram. It is likely to be about $\varepsilon = 0.2$.

Reference.

Comét, Stig and H. Lagerlöf: A mathematical analysis of indicator dilution curves leading to direct calculation of the systolic and diastolic volumes of the left ventricle and auricle and of the pulmonary blood volume: Acta Physiologica Scand. 1956. *36*. 337.

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Content and Intracellular Distribution of Adenosine Triphosphate in Cow Adrenal Medulla.[1]

By

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Cow adrenal medulla has been shown to contain adenosine triphosphate in even larger amounts than striated muscle (HILLARP, HÖGBERG and NILSON 1955). In seven experiments the ATP content was found to be about 500 mg per 100 g wet weight of medulla. This surprisingly high content suggests that ATP does not participate only in the synthetic functions of the medullary cell but maybe is involved in the storage and release mechanism of the sympathomimetic amines which are stored in large amounts in specific cytoplasmic granules (HILLARP and NILSON 1953, 1954, HILLARP, LAGERSTEDT and NILSON 1953, HILLARP, HÖKFELT and NILSON 1954, BLASCHKO and WELCH 1953, BLASCHKO, HAGEN and WELCH 1955). In view of this possibility, it seemed of interest to study the intracellular distribution of ATP — together with other acid-labile phosphates and inorganic phosphate — in the medullary cell.

Material and Methods.

To every experiment adrenal glands from 4—8 cows were used. The glands were removed from the body 40—60 minutes after the killing of the animals and immediately cooled by ice. After 1—2 hours the glands were cut in 2—3 mm thick slices and the medulla was dissected free from cortical tissue.

[1] Aided by a grant from Statens Medicinska Forskningsråd.

Extraction procedure: The weighed medulla was homogenized in trichloroacetic acid (TCA) (final concentration 5 %) with a Waring blendor for 15 seconds. After centrifugation the residue was extracted twice with 5 % TCA and the extracts combined. All operations were made at 0° C. — If the P-analyses could not be performed immediately, the extracts were neutralized with KOH to pH 5—6 and stored at — 10° C. No breakdown of acid-labile phosphate was observed for 24 hours — the longest time used — at these conditions.

Determination of P: Inorganic P (P_0), acid-labile P (P_8) (hydrolysis in N HCl for 8 min. at + 100° C) and total P (P_T) were determined after the FISKE-SUBBAROW method according to LEPAGE (1951) or in some experiments according to MARTIN and DOTY (1949). — No search has been made for the existence of very labile phosphates. The P_0-fraction may thus contain such phosphates, but the P_0-values were the same whether or not the samples were incubated with the sulphuric acid-molybdate reagent for 20 min. before adding the reducing agent.

Determinations of pentose and ultraviolet absorption were made as in a previous paper (HILLARP, HÖGBERG and NILSON 1955).

Determination of adrenaline + noradrenaline[1]*:* The total amount of these amines was determined colorimetrically according to EULER and HAMBERG (1949).

Barium fractionation of the extracted phosphorus compounds: In order to get a preliminary separation of the extracted phosphates and a removal of the catechols interfering at the following chromatography, the TCA-extracts were in two experiments fractionated (LePAGE 1951) in Ba-insoluble and Ba-soluble-alcohol-insoluble compounds and in seven experiments these two fractions were precipitated together. The precipitated compounds were dissolved in 0.1 N HCl, the barium removed with H_2SO_4 and the solution neutralized with KOH. — All operations were performed at 0° C.

Ion exchange chromatography: The solutions of Ba-precipitated phosphate compounds were brought to about pH 8.5 with ammonium hydroxide and the phosphates were adsorbed on a Dowex 2 column (200—400 mesh, chloride form, 40 × 13 mm, flow rate 0.5 ml/min.). They were eluted according to the method of COHN and CARTER (1950) (two experiments) or according to the modification of this method by DEUTSCH and NILSSON (1953). Fractions of 25 ml volume were collected at a flow rate of 1 ml/min. and analyzed for P_0, P_8, P_T, pentose and ultraviolet absorption.

In two experiments no Ba-precipitation was made but the sympathomimetic amines were instead removed by a cation exchanger. The medulla (5 g) was homogenized in 10 ml 0.6 N perchloric acid (PCA) and reextracted twice with 5 ml 0.4 N PCA. The combined extracts were neutralized with KOH to pH 6—6.5 and left at 0° C. for 40 min. The precipitated potassium perchlorate was separated by centrifugation and washed once with 2 ml water. The extract was made to 50.0 ml with water, and 48.0 ml were filtered through a Dowex 50 column

[1] For simplicity's sake the term 'catechols' is used for adrenaline + noradrenaline.

(50—100 mesh, hydrogen form, 85 × 13 mm, 1 ml/min.). The column was washed with 5 × 10 ml water. The catechols were removed but all P-containing compounds recovered. The effluent was brought to pH 8.5 with ammonium hydroxide, and the phosphorus compounds were adsorbed on a Dowex 2 column (53 × 13 mm) and eluted according to DEUTSCH and NILSSON (1953).

Cell fractionation: The medulla was homogenized in 0.3 M sucrose as described in a previous paper (HILLARP, LAGERSTEDT and NILSON 1953). Unbroken cells and nuclei were removed by centrifugation at 800 x g for 6 min. The sediment was washed once with sucrose and the combined supernatants (H) were separated into two fractions by centrifugation (6,800 x g, 30 min., + 4° to + 8° C): the catechol containing granules (G) and a supernatant (S) which thus contained all the remaining cytoplasm. Generally the granules were washed once with 0.3 M sucrose.

By determinations of the catechol content in the unfractionated medulla and in the various fractions, it was possible to calculate from which amount of medulla the fractions H, G and S were derived. — The degree of homogenization was 70—80 %, and the isolated specific granules generally contained 75—84 % of the total catechol content in the different homogenates.

Determinations of P were made after TCA-extractions of the various fractions.

Experimental Results.

I. The medullary content of P_0, P_s, P_T and ATP.

As can be seen from Table I, there are very large amounts of total phosphorus in the adrenal medulla. The mean value of eleven determinations was 207 mg P_T per 100 g wet weight. The P_0-fraction was found to be 28 % (57.0 mg/100 g) and the acid-labile P 38 % (79.5 mg/100 g) of P_T. As there are reasons (see Section II) to believe, however, that about 12 % of the P_s-fraction has broken down *before* the extraction of the medulla, the P_0-fraction probably is about 16 % too high. Some very labile phosphate esters may have been split during the extraction and P-determination procedures and thus also are included in the inorganic P-fraction.

The results of the ion exchange chromatography of the phosphorus compounds are found in Table II. The recovery of P_0 was 94—103 % and of P_s 94—100 %. The main fraction of acid-labile phosphates (83—93 % of P_s) was eluted with 0.01 N HCl—0.2 M NaCl and has in a previous paper (HILLARP, HÖGBERG and NILSON 1955) been identified as ATP (two-dimensional paper chromatography and countercurrent distribution). The ATP

content in the nine experiments performed varied between 400 and 595 mg ATP (free acid) per 100 g wet weight of medulla and the mean value was 505 mg/100 g (calculated on the basis of the phosphorus contents).

In seven (III—IX) of the nine experiments Ba-precipitation or Ba-alcohol-precipitation of the phosphorus compounds were performed before the ion exchange chromatography. As can be seen from Table I, these procedures did not result in a complete precipitation of the inorganic P. It may thus be suspected that also the acid-labile phosphates were incompletely precipitated. In the last two experiments (X—XI in Table II) no such precipitation was made but the interfering catechols were removed by a cation exchanger. The total amounts of extractable phosphorus compounds were consequently chromatographed.

As seen from Table II and Fig. 1, a P_s-fraction (6—13 % of the total P_s) was eluted immediately after the inorganic P. The ultraviolet absorption and the molar ratios pentose : P_s : P_T of this fraction suggested that the eluted compound was adenosine diphosphate (ADP). If this is the case, there are appreciable amounts of ADP in the medulla. Calculated on the basis of phosphorus contents, 50—135 mg ADP (free acid) were found per 100 g wet weight.

As evident from the analyses of the eluted fractions, adenosine monophosphate (AMP), if present in the adrenal medulla, must be sought in the first fraction just preceding and probably contaminating the P_0-fraction (Fig. 1). However, the main compound cannot be AMP as calculations on the basis of the ultraviolet absorption gave considerably lower values than calculations based on the phosphorus and pentose determinations. As furthermore the amount of P_T in this fraction constituted only 1—3 % of the total amount of P_T, the content of AMP in the medulla must be very low as compared to the content of ATP and ADP (?).

A small fraction of P_s (3—5 % of the total P_s) was constantly eluted with 0.01 N HCl—0.05 M NaCl. The analyses did not give any suggestions as to the nature of this compound. Continued elution with this solution resulted only in the appearance of ATP.

II. The intracellular distribution of P_0, P_s, P_T and ATP.

To get a general picture of the distribution of the phosphorus compounds in the medullary cells, four cell fractionations were

Table I.

The content of P_0, P_s and P_T in cow adrenal medulla (μg per g wet weight).

	Catechol content mg/g	P_0		P_s		P_T
		μg/g	Per cent of P_T	μg/g	Per cent of P_T	μg/g
I	11.4	595		790		
II	12.0	565	30	775	42	1,860
III Total		495	26	765	40	1,890
III Barium-insoluble		405		680		1,390
III Barium-soluble alcohol-insoluble		60		46		189
IV Total		480	25	795	42	1,890
IV Barium-insoluble		395		600		1,370
IV Barium-soluble alcohol-insoluble		48		45		166
V Total	14.4	655	28	960	41	2,330
V Barium-insoluble Barium-soluble alcohol-insoluble		555		805		2,000
VI Total	12.6	555	25	785	36	2,210
VI Barium-insoluble Barium-soluble alcohol-insoluble		525		730		1,780
VII Total	15.1	570	26	740	34	2,170
VII Barium-insoluble Barium-soluble alcohol-insoluble		540		625		1,590
VIII Total		480	23	890	43	2,070
VIII Barium-insoluble Barium-soluble alcohol-insoluble		430		810		1,810
IX Total		560	32	610	35	1,750
IX Barium-insoluble Barium-soluble alcohol-insoluble		500		555		1,390
X	12.3	600	28	830	38	2,170
XI	12.6	740	32	785	34	2,330
Average:	12.9	570	28	795	38	2,070

Table II.

Ion exchange chromatography of the acid soluble phosphorus compounds according to Cohn and Carther (1950) (experiments III and IV) or according to the modification of this method by Deutsch and Nilsson (1953).

The chromatography was performed on the Ba-insoluble (III—IV), the Ba-insoluble and alcohol-insoluble (V—IX) or the total acid soluble phosphorus compounds (X—XI). In experiments X and XI the catechols were removed by a cation exchanger. — The experiments are the same as in Table I.

	Weight of medulla g	Elution with 0.003 N HCl		Elution with 0.01 N HCl 0.02 M NaCl			Elution with 0.01 N HCl 0.05 M NaCl		Elution with 0.01 N HCl 0.2 M NaCl		Recovery	ATP mg per 100 g medulla
		P_0 µg	P_s µg	P_0 µg	P_s µg	Per cent of total P_s	P_s µg	Per cent of total P_s	P_s µg	Per cent of total P_s		
III	5.60	0	0	2,130	255	6.7	—		3,550	93.5	P_0 94 % P_s 100 %	520
IV	5.60	0	0	2,230	255	7.7	—		3,050	92.5	P_0 100 % P_s 98 %	445
V	4.55	0	0	2,570	265	7.3	118	3.3	3,250	89.5	P_0 103 % P_s 99 %	585
VI	5.65	0	0	2,840	277	7.2	161	4.2	3,450	88.5	P_0 96 % P_s 94 %	500
VII	5.65	0	0	2,970	335	9.8	158	4.7	2,890	85.5	P_0 97 % P_s 96 %	420
VIII	5.65	0	0	2,410	297	6.5	151	3.3	4,110	90.0	P_0 99 % P_s 100 %	595
IX	5.65	0	0	2,730	197	6.4	111	3.6	2,770	90.0	P_0 98 % P_s 98 %	400
X	4.95	3,100	0	0	415	10.3	183	4.5	3,250	85.0	P_0 98 % P_s 98 %	535
XI	4.60	3,450	0	0	455	12.7	163	4.6	2,960	82.5	P_0 99 % P_s 100 %	525

made. The excised medulla was divided into two portions. One was extracted with TCA for determinations of the catechol, P_0, P_s, and P_T content of the unfractionated medulla. The other portion was homogenized in 0.3 M sucrose and the homogenate was separated into two fractions: the specific catechol

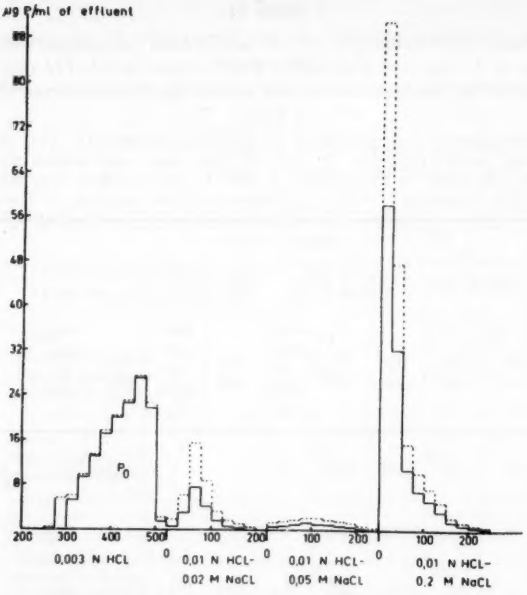

Fig. 1. Ion exchange chromatography of the acid soluble phosphorus compounds of cow adrenal medulla (4.60 g wet weight). The chromatography was performed according to the method of COHN and CARTER (1950) as modified by DEUTSCH and NILSSON (1953). The catechols were removed by a cation exchanger before the chromatography.

———— P_s or P_s. - - - - - - P_T.

containing granules (G) and a supernatant (S) containing all the remaining cytoplasm. No further fractionation of S was made, as the specific granules were the main interest in these experiments. — Nine similar fractionations were made but without analyses of the whole medulla corresponding to the homogenates (Table IV). In six of these experiments (I—VI) the granules were washed once in sucrose.

As can be seen from Table III, the main part of the acid-labile phosphates were localized to the specific medullary granules. Of the total P_s content of the unfractionated medulla 76—89 % (M = 84 %) were recovered in the granules and only about 3 % in the remaining cytoplasm. As the recovery of P_T was approximately 98 %, the loss of P_s during the cell fractionations cannot be satisfactorily explained on the basis of a loss of the specific

granules. A part of the lost P_s may be localized to the cell nuclei which were not analyzed. The main explanation seems to be, however, that some acid-labile phosphates have been broken down. This is evident from the fact that the P_o content is higher in the G + S fractions than in the unfractionated medulla.

Small and considerably variable amounts of P_s were found in the remaining cytoplasm (see also Table IV). It is difficult to decide if this P_s content has any significance as it may be an expression of a contamination from the G fraction.

As ATP represents almost 90 % of the P_s content in the medulla (Table II), the distribution of the acid-labile phosphates consequently shows that ATP to the main part must be localized to the specific catechol containing granules.

There are strong reasons to suppose that the total contents of adrenaline and noradrenaline of the cow medulla *in the living cell* are localized to the specific granules, the amounts found in the remaining cytoplasm at cell fractionations being released postmortem (HILLARP, LAGERSTEDT and NILSON 1953). When the granules are damaged in such a way, however, that the catechols are liberated, a release and a rapid enzymatic breakdown of the acid-labile phosphates in the granules takes place (HILLARP and FALCK, 1956). It may thus be assumed that in the experiments performed about 25 % of the granules — as can be seen from the catechol distribution values in Table III — have released their acid-labile phosphates. As the liberated phosphorus compounds were not recovered in the S-fractions, they must have been dephosphorylated. It follows that the P_s content of the living cell must be at least $\frac{100}{75}$ times higher than the observed values for G. Thus, it can be calculated that the observed P_s content in the unfractionated medulla is about 12 % too low and the P_o content about 16 % too high at least.

As can be seen from Tables III and IV, the content of inorganic phosphate in the unwashed granules was about one third of the content in the remaining cytoplasm. The P_o content was considerably lower in granules once washed with sucrose. In fifteen experiments (nine are not recorded in the tables) the P_o content in unwashed granules was found to vary between 16 and 27 (M = 22) μg per 1,000 μg catechols (in G), representing about 15 % of P_T. The content in granules once washed with sucrose

Table III.

The intracellular distribution of catechols, P_0, P_s and P_T in the medullary cell. The experiments are the same as in Tables I and II.

M: unfractionated medulla, G: granular fraction, containing the specific medullary granules, S: remaining cytoplasm.

Medullary fractions	Catechols mg/g medulla	P_0		P_s		P_T
		µg/g medulla	Per cent of P_T in G	µg/g medulla	Per cent of P_s in M	µg/g medulla
M:	14.4	655		960		2,330
V. G:	75 %	231	14	830	86	1,600
S:	25 %	620		34		910
M:	12.6	555		785		2,210
VI. G:	71 %	165	13	600	76	1,310
S:	29 %	640		29		865
M:	15.1	570		740		2,170
VII. G:	79 %	185	13	660	89	1,380
S:	21 %	555		6		760
M:	12.6	740		785		2,330
XI. G:	75 %	77	7.0	670	85	1,140
(washed once)						
S:	25 %	625		36		875
M:	13.7	630		815		2,260
Average: G:	75 %	165		690	84	1,360 | 2,210
S:	25 %	610		26	3	850 /

was in thirteen experiments (Tables III, IV and VI) about 7 µg, representing about 5 % of P_T. Calculations on the basis of the P_0 concentrations in S and of the volumes of G and S show that the higher values in unwashed granules cannot be explained satisfactorily as due only to a contamination of G with S. Inorganic phosphates may thus be lost from the granules to an unknown degree and their distribution *in the living cell* cannot be estimated even approximately.

The P_0 content of the washed granules expressed per 1,000 µg catechols was rather constant about 7 µg (80—100 µg per g wet weight of medulla). This small amount was found to be firmly bound to the granules. A repeated washing did not result in any significant loss (Table V).

The ATP and the other acid-labile phosphates in the granules show a remarkable stability. They were not released or split to

Table IV.

The distribution of catechols, P_e, P_s and P_T between the specific medullary granules (G) and the remaining cytoplasm (S) of nine different homogenates.

In experiments I—VI the G fraction was washed once in sucrose, in experiments VII—IX the granules were unwashed.

		Catechols		P_e		P_s	P_T
		μg	Per cent	μg	Per cent of P_T in G	μg	μg
I.	G:	4,600	80	30	4.7	350	640
	S:	1,150	20	246		6	330
II.	G:	4,350	81	30	4.8	335	620
	S:	995	19	222		4	300
III.	G:	4,500	81	31	5.1	315	615
	S:	1,020	19	223		6	305
IV.	G:	5,700	84	43	4.9	415	810
	S:	1,060	16	292		8	400
V.	G:	5,400	82	38		390	
	S:	1,170	18	250		10	
VI.	G:	5,850	84	43		420	
	S:	1,110	16	223		18	
VII.	G:	11,800	76	287	18	815	1,590
	S:	3,900	24	835		15	970
VIII.	G:	10,300	76	237	18	755	1,330
	S:	3,300	24	675		0	785
IX.	G:	7,850	90	144	14	595	1,050
	S:	925	10	470		31	620

any significant degree in granules suspended in 0.3 M sucrose and kept at + 4° C up to 72 hours (Table VI). Neither were these compounds liberated at washing of the granules (Table V).

Ion exchange chromatography of the phosphorus compounds in the specific granules was performed in one experiment (Fig. 2). An aliquot of the same medulla as in experiment XI (Table II and Fig. 1) was used. The granules were isolated in 0.3 M sucrose, washed once and extracted with perchloric acid. The extract was treated in the same way as the extract of the unfractionated medulla.

Table V.

The contents (in μg) of P_0 and P_s in medullary granules from six different homogenates.

The granules were washed once or twice in sucrose.

		1	2	3	4	5	6
P_0	One washing	30	31	31	41	35	36
	Two washings	26	26	29	37	34	36
P_s	One washing	345	325	320	415	345	385
	Two washings	340	320	320	420	345	360

A comparison between Figs. 1 and 2 shows that the ATP fraction is even more dominating in the isolated granules than in the medulla due to the fact that the other two P_s fractions are considerably lower in the granules. To make the comparison more easy, the P_s values for the three fractions containing acid-labile phosphates are given below per g wet weight of medulla.

	I	II	III
Medulla	98	35	640
Washed granules ..	37	14	630

Table VI.

The contents of P_0 and P_s in medullar granules (from six different homogenates) kept in 0.3 M sucrose 0—96 hours at + 4° C.

The granules were washed once in sucrose. The contents are expressed as μg per 1,000 μg catechols in the granules.

	0 hours			24 hours		48 hours		72 hours		96 hours	
	P_0	P_s	P_T	P_0	P_s	P_0	P_s	P_0	P_s	P_0	P_s
1	7	76	139	7	76	7	77	7	74		
2	7	77	142	7	77	7	76	7	75		
3	7	70	136	7	71	7	71	7	71		
4	8	73	142	7	73	8	75	7	73		
5	7	73		7	71	7	70	7	68	7	65
6	7	73		7	71	6	71	6	69	6	67

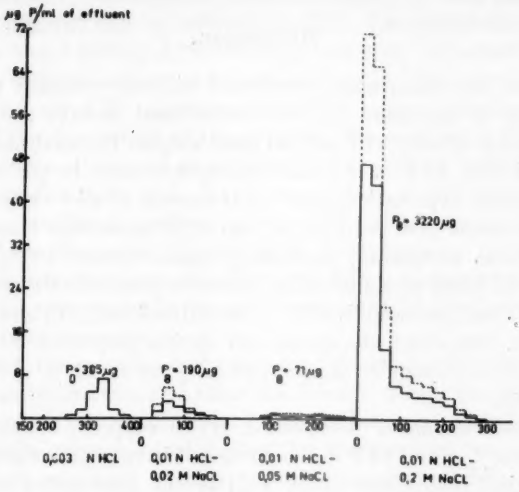

Fig. 2. Ion exchange chromatography of the acid soluble phosphorus compounds in the specific medullary granules isolated from an aliquot of the same medulla as in Fig. 1.

It can be seen, that 98 % of the ATP fraction in the medulla but only 38 % of fraction I, probably containing ADP, were found in the washed granules. The figures show, too, that more than 90 % of the P_s in the granules is represented by ATP.

The phosphorus compounds of the medulla eluted just preceding and together with P_0 (Fig. 1) could not be demonstrated in the washed granules. It can thus be concluded that no or only very small amounts of AMP were localized to these structures after washing. — It is of course at present impossible to decide whether the compounds not recovered in the washed granules originally have been located to these structures and have been released during the cell fractionation or have their localization to other parts of the cytoplasm.

As almost all of the ATP in the medulla was recovered in the granules, it may be inferred that the P_s breakdown observed at fractionation of the medullary cells to the main part is referred to other acid-labile phosphates than ATP. This is another demonstration of the high stability of the medullary ATP.

Discussion.

In the nine experiments performed with ion exchange chromatography it was found that the cow adrenal medulla contains on the average 505 mg ATP per 100 g wet weight. The analyses further showed that ATP is the main phosphate ester in the medulla. Contrary to expectation, AMP — if present at all — can amount to only some few per cent of the ATP quantity. However, a phosphorus compound, containing approximately 10 % of the total acid-labile phosphates, is constantly present in the medullary extracts and probably is ADP on account of its P_s, P_T and pentose content, ultraviolet absorption and elution properties. This being the case, the medulla may contain ADP up to 20 % of the ATP amounts.

There are reasons (see Section II) to suppose, however, that about 25 % of the ATP in the living cell have broken down before extraction could take place. This is not unexpected since the adrenal glands used in this work remained *in situ* for 40—60 minutes before being removed and chilled. The real content would thus be almost 700 mg/100 g. As it has been found (HILLARP and FALCK 1956), that the ATP breakdown in homogenates of cow medulla does not go further than to AMP, this compound might be expected in extracts of the medulla. A breakdown of ATP amounting to 25 % would give about 110 mg AMP/100 g medulla. Such large amounts, however, have not been recovered. It is possible that the AMP, too, has been dephosphorylated in spite of the fact that no such process has been detected in homogenates. But the explanation may rather be that only one phosphate group has been split off from ATP. This would give about 140 mg ADP/100 g. This amount is in good agreement with the quantities recovered in experiments X and XI (Table II) in which 115 and 135 mg of ADP (?) were found. (As pointed out in Section I, the lower recoveries in the other experiments may be due to incomplete Ba-precipitation.) Thus, the observed ADP (?) content in the medulla may be only a result of ATP breakdown.

As well the experiments on the distribution of acid-labile phosphates within the medullary cell as the ion exchange chromatography of the specific granules showed that ATP almost exclusively is localized to the catechol containing granules. The P_s content of washed granules was on the average 74 μg/1,000 μg

catechols and — as shown by chromatography — more than 90 % of this amount can be referred to ATP. The granules can thus contain about 550 μg ATP/1,000 μg catechols. Calculated on the basis of determinations in a previous paper (HILLARP and NILSON, 1954 a), ATP may amount to 275 μg/mg proteins and to 9 % of the dry weight of the granules. The content is thus very considerable, above all as compared with that of mitochondria. BRENNER-HOLZACH and RAAFLAUB (1954) found about 4 μg ATP/mg proteins in rat liver mitochondria, which is in good agreement with the value for acid-labile phosphates in mouse liver mitochondria (KIELLEY and KIELLEY 1951). A somewhat higher value for these phosphates was determined by CLELAND (1954) in cat heart sarcosomes.

It may be questioned if the values found for the content of inorganic phosphates and their distribution within the medullary cell have any real significance. An unknown amount of phosphate from labile esters are included in this fraction and the inorganic phosphate may have changed place during the cell fractionation. Furthermore, the rather constant content of inorganic P found firmly bound to the granular fraction is so low that it may well be localized to other structures in this fraction contaminating the specific granules. Contaminating particles, *e. g.* mitochondria, have not been found (cf. HILLARP, LAGERSTEDT and NILSON 1953), but small amounts of such structures cannot be excluded as yet.

It is at present unknown how the large amounts of adrenaline and noradrenaline are stored in the medullary granules. The concentration of the sympathomimetic amines is about 0.25 M, *i. e.* twice the concentration of total bases in serum. As the granules can be kept in 0.25—0.3 M sucrose at + 4° C for long periods (48—72 hours) without swelling, catechol release or ATP breakdown, it may be inferred that the amines are more or less osmotically inactive. They may be bound to proteins or solved in a lipid phase, but already quantitative considerations make these possibilities improbable (0.6 and 0.8 mg catechols per mg proteins and lipids respectively). Evidence speaking against this assumption was also obtained by paper electrophoresis (HILLARP and NILSON, 1954 b). The rapid release of the stored catechols, for instance at transferring the granules to hypotonic solutions, and the high solubility in water suggest, however, that adrenaline and noradrenaline exist in a soluble form within the granules.

It has been suggested (KENDALL 1932, UTEVSKI 1936) that adrenaline is stored bound to lactic acid. CRAWFORD (1951) has shown, however, that the lactyl adrenaline found in medullary extracts is an artefact. Furthermore, less than 10 % of the lactic acid in the cow medulla are localized to the granules, the concentration being less than 0.04 M/M catechols (unpublished observations).

As the catechols in the granules seem to be more or less osmotically inactive, it may be assumed that they are kept in a bound form. Complex formation with a polyvalent anion might be a possibility to reduce the osmotically active substances in the granules. The high amount of ATP is interesting from this point of view.

The ATP in the catechol containing granules may have the same functions as in other cells. It is, however, difficult to understand why the not very active medullary cell with its presumably rather uncomplicated synthetic reactions should contain so much higher ATP content than other cells. The very large amounts of ATP and the very low contents of AMP and probably of ADP, the high stability of ATP in granules without active respiration, and its wellknown property to form complexes with cations may suggest that it is involved in the storage and release mechanism of adrenaline and noradrenaline. It is from this point of view tempting to speculate on the possibility that the amines are stored as complexes with ATP. The specific granules do not leave the cell at secretion but the sympathomimetic amines are liberated and probably rapidly transported through the cytoplasm out of the cell (HILLARP, HÖKFELT and NILSON 1954). When the catechols are released, the energy of ATP might be used for their transport out of the cell. Such a function of ATP is not quite unreasonable considering the special demands on medullary secretion. At certain circumstances the medulla is put into high activity with secretion of large amounts of the stored amines in a short time. This large and rapid output may be assumed to require considerable quantities of energy in a ready available form. For this purpose the ATP stored in the granules would be convenient.

Summary.

In nine experiments performed with ion exchange chromatography of the acid soluble phosphorus compounds in the cow adrenal medulla it was found that this tissue contains on the average 505 mg ATP per 100 g wet weight. There are reasons to suppose, however, that about 25 % of the ATP in the living cell have broken down before extraction could take place. The real content would thus be about 700 mg/100 g.

The analyses further showed that ATP is the main phosphate ester in the medulla. AMP — if present at all — can amount to only some few per cent of the ATP quantity. The medulla may contain ADP up to 20 % of the ATP amount but there are reasons to believe that the observed ADP (?) content may be only a result of ATP breakdown.

As well experiments on the distribution of acid-labile phosphates within the medullary cell as ion exchange chromatography of the phosphorus compounds in the specific granules of the medullary cell showed that ATP almost exclusively is localized to these granules containing the sympathomimetic amines. No AMP and only small amounts of ADP (?) and of inorganic P were found in granules isolated and washed in 0.3 M sucrose.

The content of acid-labile phosphorus in washed granules was on the average 74 μg/1,000 μg catechols and more than 90 % of this amount can be referred to ATP. The granules can thus contain about 550 μg ATP/1,000 μg catechols (about 9 % of the dry weight of the granules).

The ATP in the medullary granules shows a remarkable stability. It is not released or split to any significant degree in granules suspended in 0.3 M sucrose and kept at + 4 C up to 72 hours.

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An Attempt to Condition Hypothalamic Polydipsia.

By

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It has earlier been shown that osmotic (ANDERSSON 1952, 1953) and electrical (ANDERSSON and MCCANN 1955 a and b, GREER 1955) stimulation of a certain area of the hypothalamus can cause polydipsia. The opposite effect, hypodipsia and adipsia has been seen as a result of lesions in the hypothalamus (STEVENSON 1949, STEVENSON, WELT and ORLOFF 1950, WITT, KELLER, BATSEL and LYNCH 1950, ANDERSSON and MCCANN 1956). Taken together these two findings strongly indicate the presence of an essential drinking centre in the hypothalamus. So far, however, nothing seems to be known about the way in which the urge to drink reaches consciousness and about eventual cortical representation of the thirst sensations. With this in mind it seemed to be of interest to study if it were possible to develop a conditioned "drinking reflex" on the basis of hypothalamic polydipsia induced by electrical stimulation. Especially for two reasons the goat was a suitable animal for such a study. Here the polydipsic effect of electrical stimulation of the "drinking area" of the hypothalamus seems to be repeatable practically at will and the presence of a voluminous rumen, which takes up most of the water drunk by the animals, seems to make the temporary inhibition of thirst from the alimentary canal much less pronounced than it is in non ruminant species (ANDERSSON and MCCANN 1955 a).

Methods.

Five fullgrown female goats were used for the experiments. The electrical stimulations were performed using HESS' technique (1932, 1949) somewhat modified for the use in goats (ANDERSSON 1951). The parameters of stimulation were 0.5—1 V and 50 c/s.

As soon as repeated preliminary stimulations at a certain point in the hypothalamus had been found to cause obvious polydipsia, indicating that the tip of the effective electrode was situated within the "drinking area" of the hypothalamus, the animals were fixed in a Pavlov stand with a water basin within comfortable reach. The Pavlov stand in its turn was standing within a pen with 1.4 m high, closed walls. Thus the animals were during the experiments unable to watch the experimenter, who on his hand observed the behavior of the animals by help of a mirror placed behind the Pavlov stand. On the wall of the pen just in front of the goat's head was a signal lamp, the lightening of which was used as one of the neutral stimuli tried during the experiments. The other neutral stimuli used were a tone from a tone generator and the temporal stimulus obtained by keeping a constant time interval between the onset of each stimulation. Any other form of external disturbance to the animals was avoided as much as possible during the experiments.

The histological localization of the effective points of stimulation in the hypothalamus was facilitated by making small electrocoagulations at the tips of the electrodes before killing the animals.

Results.

During the attempts to condition the polydipsic effect of hypothalamic stimulation the goats in no case seemed to be disturbed by the experimental conditions. They were thus standing calmly in the Pavlov stand throughout the experiments and, with the exception of occasional licking, polydipsia was the only effect observed as a result of the electrical stimulations. These never failed to elicite drinking of water and the animals continued to drink without interruption during the fairly short periods of hypothalamic stimulation and for 2 to 3 seconds afterwards. The time of latency before the onset of drinking was short, generally 3 to 5 seconds, but could during the first five stimulations in an experiment be a little longer, up to 10 seconds. The interval between the stimulations was in each experiment kept constant and in order to keep down the amount of water drunk during each period of electrical stimulation these never exceeded 15 seconds.

When combined with electrical stimulation the neutral stimuli were always covering the electrical stimulation in time, thus starting 1, 2 or 3 seconds before and ending 2 or 4 seconds after the electrical stimulation. These time intervals were kept constant in one and the same experiment.

Neutral stimuli alone were tried for the first time after 30 to 50 constellations of neutral stimuli and electrical stimulation but did never at this point or later in an experiment cause the animals to drink or make them show even the slightest interest in the water offered. The goats, however, always paid obvious attention to the light and sound signals.

The points in the hypothalamus where the electrical stimulation produced drinking of the animals were in these experiments all located within the "drinking area" described earlier (ANDERSSON and MCCANN 1955 b).

An example of one of the experiments is given in the following protocol.

Exp. 2. Neutr. stimuli: Light and tone started 2 secs. before and continued for 4 secs. after the el. stim.
Duration of el. stim.: About 10 secs.
Duration of simply neutr. stimuli: 30 secs.
Interval between the onset of each period of stimulation: 3 min.

Number of stimulations	Kind of stimulus	Drinking effect
35	El. stim. and neutr. stim.	positive
1	Neutr. stim.	negative
43	El. stim. and neutr. stim.	positive
1	Neutr. stim.	negative
32	El. stim. and neutr. stim.	positive
1	Neutr. stim.	negative
1	El. stim. and neutr. stim.	positive

During these stimulations the goat drank 7.5 liters of water.

At the termination of the actual conditioning experiments none of the goats was overloaded with water as shown by the fact that they could easily be brought to drink several liters of water more during a finishing prolonged period of electrical stimulation. In all of the five animals tested it was sufficient to combine the neutral stimuli five times with a moderate electric chock applied

subcutaneously in one hind leg and causing a pain reaction in order to obtain a similar reaction for solely the neutral stimuli in question.

Discussion.

MASSERMAN's attempts (1943) to condition the "shame rage" reaction produced by electrical stimulation in the hypothalamus of cats seem to be the only previously reported experiments where it has been tried to condition the effect of direct hypothalamic stimulation. In his experiments 60 to 480 constellations of neutral stimuli and electrical stimulations producing "shame rage" in none of 18 cats resulted in any manifest rage reactions to solely neutral stimuli. MASSERMAN suggested as the most likely explanation to this negative finding that the direct hypothalamic stimulation had not produced any significant conative or affective state of the animals, and that there thus was little or no basis for the thesis that the hypothalamus governs or even mediates the emotional experiences themselves.

Even if this might be the case as concerns emotional experiences there are good reasons for believing that the polydipsia seen as a result of hypothalamic stimulation in the goat is the result of thirst feelings experienced by the animals. Thus when stimulated electrically or osmotically within the "drinking area" of the hypothalamus the goats go for the water by themselves and find it even if the water basin should be placed in the opposite corner of the pen. If, as can be expected from this behavior of the animals, the stimulation of the "drinking area" of the hypothalamus gives rise to impulses which in some way reach the cerebral cortex and there manifest themselves as a conscious urge to drink, at least one pre-requisite for the development of a conditioned reflex of classic Pavlovian type (PAVLOV 1927) seems to have existed in the experiments described in this paper.

However, the attempts to condition the hypothalamic polydipsia were completely fruitless. Many, more or less likely, explanations of the failure can be suggested and the result does not exclude that it still might be possible to condition hypothalamic polydipsia. Thus the neutral stimuli may not have been strong enough for the development of a conditioned reflex, especially as one has to take into consideration an eventually gradually increasing, local inhibition of thirst from the alimentary canal

during the course of the experiments. But the facts that the effect of the electrical stimulations did not seem to be weakened towards the end of the experiments and that the animals at the termination of the actual conditioning experiments always easily could be forced to drink additional large amounts of water during prolonged stimulation indicate that a local inhibition of thirst from the alimentary canal has not been very intense even towards the end of these experiments. It can further be argued that goats might not be suited for conditioning experiments of this kind. But their learning ability seems to be fairly good and it was sufficient in the animals used for this study to combine the neutral stimuli a few times with a pain stimulus to get a similar pain reaction for simply the neutral stimuli.

The absence of a physiological basis for the conditioning of thirst sensations might however also be the reason for or contribute to the failure to condition hypothalamic polydipsia reported here. There is thus nothing indicating that the feeling of thirst has any special cortical localization, on the contrary, the removing of the greater part of the cerebral cortex or frontal lobectomy does not seem to abolish drinking in the sheep (CLARK 1953) and it has never been reported that the removal of any special part of the cerebral cortex causes adipsia. Providing the hypothalamus is functioning normally it thus seems as if an animal should be able to experience thirst if only so much of higher brain centres are left intact that consciousness is still present. Furthermore it is not known that the stimulation of any part of the brain outside the hypothalamus can cause polydipsia and there are consequently so far no evidences for the existence of *stimulatory* fibers running from other parts of the brain to the hypothalamic "drinking centre". If then the hypothalamus is the only place in the body from where a real thirst feeling can be elicited and the "drinking centre" there can not be stimulated from higher organized parts of the brain, there does not seem to be any pre-requisite for a conditioning of hypothalamic polydipsia.

Summary.

Attempts were made to condition the polydipsic effect of electrical stimulations within the "drinking area" of the hypothalamus of 5 goats. In spite of from 46 to 110 constellations of neutral

stimuli and electrical stimulations the neutral stimuli alone never caused the animals to drink or made them show any interest in the water offered, although the electrical stimulations combined with neutral stimuli never failed to evoke drinking of water after a very short time of latency.

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Cholinesterases of Mice in Relation to Sex and Age.

By

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It is generally presumed that cholinesterase is related to sex in some species. Thus the cholinesterase activity of serum from mature rats and mice is far higher in the female than the male (BEVERIDGE and LUCAS 1941). SAWYER and EVERETT (1946 and 1947) have made a study of the cholinesterase activity in organs of the rat in relation to sex. They state that female serum hydrolyzes acetylcholine five times faster than that of the male, and ten times faster when benzoylcholine is the substrate. The cholinesterase level in liver and serum was raised by administering oestrogen to castrated rats of either sex, while it was lowered by testosterone. CARIDROIT et al. (1945) found fifty per cent higher serum activity in the cock than in the hen. WATTENWYL and coworkers (1943) claim that the cholinesterase activity of man passed the age of puberty is related to sex. JONES and MCCANCE (1949) examined the cholinesterase activity in the serum from the umbilical cords of new born infants. The values increased immediately after birth from a level which was about 65 per cent of the adult to around 90 per cent during the next three weeks. HUTCHINSON and WIDDOWSON (1952) measured the cholinesterase of the serum in children of either sex between the ages of six and fifteen. At the age of six the activity is 25 per cent higher than the adult level, but it decreases during the following nine years down to the adult level. They failed, however, to determine any significant difference between boys and girls.

In the work here presented a systematic study was made of the cholinesterase activity in the blood, liver and brain of mice, in relation to sex and age.

Experimental.

Methods.

Each analysis was performed in pooled samples from ten mice. The figures representing the three months old mice are the results of samples from about 30 animals. The mice were decapitated and bled. The heparinized blood was separated into plasma and erythrocytes by centrifugation, and the latter washed three times with saline. Haematocrit values were measured.

Ten per cent homogenates of brain (cerebral hemisphere) and liver (median lobe) were prepared in Ringer's bicarbonate buffer solution at 0—5° C. (Buffer solution: 0.115 m NaCl, 0.025 m $NaHCO_3$, 0.0025 m $CaCl_2$ and 0.0026 m KCl.)

The cholinesterase activity was measured by means of the standard manometric technique of Warburg at 37° C and pH 7.4 (AUGUSTINSSON 1948). Acetyl choline (0.014 m) was used as substrate. In one experiment 0.014 m acetyl-beta-methyl choline was used. The reticulocytes were demonstrated by the "wet" method of staining with the aid of a saturated solution of brilliant cresyl blue in absolute methyl alcohol. The count of reticulocytes is the average of ten mice at a time; one thousand erythrocytes being examined from each animal.

The cholinesterase activity, Q_{Ach}, is expressed as the number of microliters of carbon dioxide evolved per hour per mg wet weight, or per microliter plasma (or erythrocytes).

Results.

Plasma.

The results are given in figure I. It should be noticed that the variability was the least in plasma.

The values presented in the figure are the result of a correction to fifty per cent haematocrit. The reason for this correction is that the haematocrit value increases with age. Thus values about 60 per cent of the mature mice were observed in the infantile ones. Besides small variations were found in mice of the same age.

The levelling off of the curve was coincident with the cessation of suckling. The sex difference developed coincident with sexual maturation and was ultimately about forty per cent. It is of interest to observe the plateau established in the female, in contrast to the fall to a lower plateau in the male.

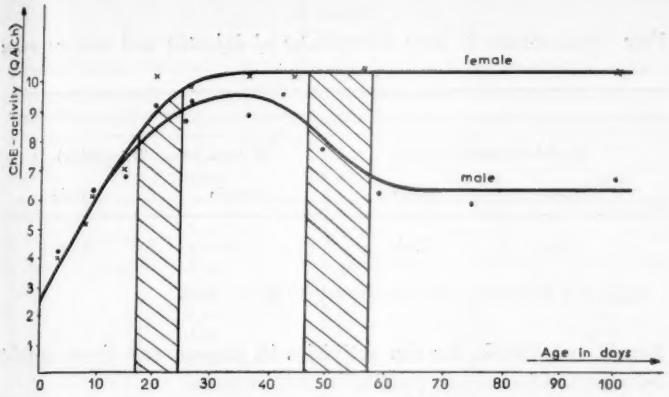

Fig. I. Relation between age and cholinesterase activity of plasma from mice of either sex.

The first change in the curve coincides with the cessation of suckling (shaded part).

The descending part of the curve for the males coincides with fertility (shaded part).

Liver.

The results are given in table I. The variability was considerable and somewhat irregular.

It appears that the values for the females were consistently higher than for the males. However, it remains to be established if the somewhat lower values in the sexual mature animals implies a decrease with age. The low values coincident with cessation of suckling may indicate a biological validity.

The simultaneous high liver and low plasma values in infantile mice is contrary to expectation, since pseudo-cholinesterase, being

Table I.

Cholinesterase values of mouse liver homogenates in relation to age and sex.

Age (days)	Q_{ACh}		Age (days)	Q_{ACh}	
	Female	Male		Female	Male
2	31.5	28.5	32	22.0	20.0
5	29.0	29.5	39	26.5	22.5
8	30.0	27.0	52	25.0	22.0
11	26.0	24.0	68	25.0	17.5
20	27.5	23.0	90	21.0	16.5
22	16.0	18.0			

Table II.

True cholinesterase in liver homogenates of infantile and mature mice of either sex.

Q_{MeCh}			
Immature mice (3 days)		Mature mice (3 months)	
Female	Male	Female	Male
1.00	1.05	0.50	0.70

$Q_{MeCh} = 1.00$ corresponds approximately to $Q_{ACh} = 2.00$.

largely responsible for the activities in plasma and liver, might be expected to vary in parallel in both organs.

As the liver activities in the early days of life might be caused by a production of true cholinesterase, the activity was measured in infantile and adult mice with acetyl-beta-methyl choline as substrate (MENDEL, MUNDELL and RUDNEY 1943).

No significant difference was observed, and the values were quite low, as seen from table II.

Brain.

Table III seems to imply a remarkably low cholinesterase activity in infantile mice. Values only 15—20 per cent of those in mature mice were obtained with three days old mice. Apparently there is a steady increase for about four weeks, until the level in mature mice is reached.

No difference due to sex could be detected.

Erythrocytes.

On account of the small samples available and the relatively low activity in erythrocytes, no analyses were obtained during the first fifteen days after birth.

The material indicates that there is a slight fall in the activity with increasing age the first month of life.

The average count of reticulocytes in mice lies between 22 and 30 per cent in 10—20 days old mice, 10—15 per cent in one month old mice and 4.5—5 per cent in three months old ones. Thus the reticulocytosis falls off with age.

It is doubtful whether there is a difference in activities due to sex.

Table III.

Cholinesterase values in brain homogenates and erythrocytes of mice in relation to age and sex.

Age (days)	Brain		Erythrocytes	
	Female	Male	Female	Male
3	1.90	1.25		
8	4.00			
9	3.80	3.55		
15	7.20	6.60		0.49
20	8.30	8.10	0.40	0.61
26	10.30	8.90	0.38	0.31
36	8.05	10.15	0.31	0.30
44	9.50	12.20	0.27	0.31
49		9.90		
56	10.60	9.80	0.29	0.34
74		9.70		0.36
100	11.60	11.40	0.26	0.30

Discussion.

The contrast between the high values in the liver and the low ones in the plasma at an early age is striking in view of the fact that the liver is considered to be the organ where plasma-cholinesterase is produced (ANTOPOL et al. 1938, FABER 1943, SAWYER and EVERETT 1947). As the content of true cholinesterase is negligible, a nonspecific esterase might be responsible. It might also be that the pseudo-cholinesterase is accumulated in the liver before it is released.

The decrease in the cholinesterase activity of plasma when the male becomes fertile implies that sex hormones exert an influence. Similar conditions have been demonstrated for rats by SAWYER and EVERETT (1946). But the difference seems to be less for mice.

Infantile mice exhibit extremely low activities in brain tissue, but somewhat higher activities in erythrocytes than do the mature ones, even though it might be expected that both organs contain predominantly true cholinesterase.

The cholinesterase values for erythrocytes and the count of reticulocytes are both higher in 20 days old mice than three months old ones. PRITCHARD (1949) has shown that an increased reticulocytosis in rats, following repeated hemorrhage, induced very high cholinesterase activities in the erythrocytes.

Summary.

1. The cholinesterase activities of liver, brain and blood of mice, and the relation to age and sex, has been studied.
2. The plasma values increased till cessation of suckling. The level was maintained in the female but was reduced by about 40 % in the male at sexual maturation.
3. Also the liver values exhibited a sex difference, most pronounced after sexual maturation. Contrary to the plasma, the activities were quite high in infantile mice.
4. The brain tissue presented extremely low values in infantile mice. No difference due to sex was established.
5. The somewhat high erythrocyte values in young mice may probably be due to a larger reticulocytosis in the same period.

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